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PHYSIOLOGICAL ZOÖLOGY

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DEVELOPMENTAL PATTERN IN THE STARFISH PATIRIA MINIATA, AS INDICATED BY INDOPHENOL

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EARLIER papers have shown that certain features of physiological developmental pattern can be rendered directly visible by differential intracellular formation, reduction, and reoxidation of the intensely blue indophenol in living, intact developmental stages of various forms. Data are at hand on early stages of the echinoid *Dendraster excentricus* (Child, 1941*b*), embryonic development and gills, legs, and balancers of larval stages of the urodele *Triturus* (Child, 1942*b*; 1943*a*, *c*), embryonic stages of the teleost *Oryzias latipes* (Child, 1942*b*, 1943*d*), and living ovaries of *Drosophila hydei* (Child, 1943*b*). The present paper is a study of those features of developmental pattern made visible by indophenol in *Patiria miniata*.

USE OF INDOPHENOL WITH *Patiria* MATERIAL

Material.—The large eggs and beautifully transparent developmental stages of *Patiria*, a form abundant at Pacific Grove, California, are extremely favorable material for work with indophenol. Data concerning oöcytes and early cleavages were obtained at Pacific Grove; and fertilized eggs transported to Palo Alto developed normally and lived for almost a month, providing material for study of later stages. I am again deeply indebted to the staff of the Hopkins Marine Station for material and facilities provided. In connection with this material, various procedures and limitations in the use of indophenol as an indicator of features of physiological pattern must be discussed somewhat more fully than they have been in earlier papers.

Concentrations of reagents and intracellular reaction.—The intracellular formation of indophenol from sea-water solutions of the reagents para-aminodimethylaniline (dimethyl-paraphenylenediamine) and α-naphthol is catalyzed by an oxidase, now regarded as cytochrome oxidase. The aniline is rather highly toxic, α-naphthol less so, to echinoderm material. Intracellular indophenol also becomes toxic and finally lethal with continued formation, but the intracellular reaction occurs rapidly with concentrations of reagents so low that reduction after reaction and reoxidation after reduction, often repeated reduction and reoxidation, are possible in the living material before the pattern is altered by differential toxic effects.

Concentrations of reagents found by

experiment to be satisfactory are as follows: For all stages except oöcytes and early cleavages, one small drop (40 drops = 1 ml.) of the Eastman Company's liquid aniline in 10 ml. of sea water and 1 mg. of α-naphthol in 10 ml. of sea water served as stock solutions and were made daily or oftener. After a number of weighings, the α-naphthol was estimated as approximately 1 mg. or less, as it became evident that the pattern of reaction was not altered by wide variations in concentration. For the reaction in developmental stages, from 1 to 5 small drops (40 = 1 ml.) of each solution were used in a total volume of 1 ml. or were simply added to 1 ml. of water containing the starfish material. The actual concentrations of pure aniline in these solutions are somewhat uncertain. If the original liquid sample is 100 per cent pure aniline, these experimental aniline concentrations range from approximately 1/16,800 to 1/3,600. These are twenty times the concentrations given in earlier papers for the same dilutions of the original liquid. Those concentrations were based on the mistaken belief that the liquid sample was approximately a 5 per cent solution. A note concerning this error and calling attention to certain other points concerning actual aniline concentrations in different samples and in experimental solutions appears elsewhere in this number of *Physiological Zoölogy* (Child, 1944). There it is pointed out that, in view of the variation in different samples of the reagent, its gradual deterioration through oxidation or otherwise, and the gradual oxidation of experimental dilutions, actual concentrations of the effective reagent may vary widely in the same volume concentration from the liquid sample.

The error as regards aniline concentrations does not involve α-naphthol concentrations as given in the earlier papers, nor does it alter the significance of the reaction, reduction, and reoxidation patterns; these remain essentially the same except as regards time of appearance and rate of progress of the reaction with very great differences in absolute and relative concentrations of reagents until they become almost immediately lethal or extremely low.

For blastulae, gastrulae, and earlier larval stages approximate volume concentrations of from 1/16,000 to 1/8,400 liquid aniline and from 1/420,000 to 1/210,000 α-naphthol were usually satisfactory and most frequently used. The α-naphthol concentrations are much lower than those of aniline, because, when the data were obtained, it was still believed that the liquid aniline samples were approximately 5 per cent solutions and that the experimental concentrations of the two reagents were nearly the same. Much higher α-naphthol concentrations can be used without altering patterns. The lower of the above concentrations are referred to in the following text as standard concentrations for the *Patiria* material. With these concentrations the indophenol color becomes visible in the more rapidly reacting regions of blastulae and gastrulae within 5–10 minutes, and in 10–15 minutes more the gradient pattern is distinct. Concentrations much above these give rapid reaction, but retardation of reduction and other toxic effects appear early. The reagents pass the fertilization membrane rapidly; embryos within the membrane react almost as rapidly as those without it. Penetration into the blastocoel is also rapid, even with the low concentrations used. Brief exposure to one reagent, followed by transfer to the other, shows that differential reaction is not dependent on differential penetration.

In attempts to discover the most favorable concentrations for patterns of particular organs and parts of later larval stages, absolute and relative concentrations of reagents have been much more widely varied; but in these, as in earlier stages, the same patterns appear with a wide range of concentrations. Susceptibility to toxic effects of the reagents and of indophenol increases during development—at least to the gastrula—and becomes somewhat less in advanced larval stages. Data on oöcytes and early cleavages were obtained a year later than those on other stages, and at a time when only an old, partially oxidized aniline solution was available. However, definite, consistent results were obtained with volume concentrations three to six times the standard, and with cleavage stages one or two cleavages occurred after the reaction pattern became visible.

With nonmotile stages, frequent stirring is necessary, to avoid local differences in concentration and reduction in individuals crowded together. Actively motile stages serve as stirrers but may aggregate, with consequent retardation of reaction or with occurrence of more or less reduction.

With the use of a wide range of concentrations of reagents, in order to distinguish, as far as possible, normal physiological patterns from toxic or other possible modifying effects, intracellular indophenol reaction shows very definite and constant axial and other regional differentials and is therefore a very delicate indicator of certain aspects of developmental pattern associated with differences in oxidase activity. In all organisms examined thus far, reaction patterns of earlier developmental stages are gradients. In the later stages of some forms, certain differentiated cells show differences in reaction from others limited by cell boundaries. With increasing intracellular concentration of indophenol the reaction is apparently retarded, so that the regional differences of earlier stages of reaction become progressively less distinct, and the color may finally appear uniform. Even in these cases, however, toxic effects, such as retardation and stoppage of cilia, retardation of reduction, and finally cytolysis, are usually differential, unless concentrations of reagents are so high that regional differences are obliterated very early. Material remains in the solution of reagents during reduction and reoxidation as well as during reaction.

Intracellular reduction.—Until intracellular concentrations of indophenol become distinctly toxic, only slight oxygen decrease in the solution is necessary to bring about intracellular reduction with complete decoloration. When the reaction is regionally differential, reduction is also normally differential and, with certain exceptions noted below, progresses in the same direction as the reaction; that is, regions of more rapid reaction also reduce more rapidly than those of slower reaction until retarded by toxic effects. So little decrease in oxygen content is required for reduction that material remaining at the bottom of solutions 5–6 mm. deep on tissue-culture depression slides, with a large surface exposed to air, may undergo partial or complete reduction if left undisturbed for a few moments, particularly if slightly crowded; and local reductions may occur in regions in contact with other individuals.

As in the earlier work with indophenol, two methods of bringing about reduction have been used: oxygen decrease by oxygen uptake of considerable numbers of individuals sealed in small volumes of solution of the reagents, and by mi-

nute amounts of sodium hydrosulphite ($Na_2H_2S_2O_4$). In the sealed preparations, reduction occurs within a few minutes or more slowly, according to number of individuals and volume of solution. To insure uniform oxygen decrease in sealed preparations of nonmotile stages, the vaseline ring or depression slide must be deep enough, and the material not too crowded, to permit change of position by gravity with frequently altered position of the slide. With hydrosulphite only a few tiny granules are sufficient to bring about reduction in a few seconds in material in 1 ml. of solution. The amount is so small that weighing is impracticable, and with a little experience the approximate amount necessary can be very closely estimated; if necessary, more can be added. The amount required is not appreciably toxic; reduction can often be repeated after reoxidation on the same material without evidence of toxic effects. In sufficiently high concentrations, hydrosulphite is, of course, toxic or rapidly lethal; but in the very low concentrations used in the reduction of intracellular indophenol, it apparently does not penetrate appreciably before it is oxidized. Use of hydrosulphite makes it possible to bring about reduction as rapidly as desired in open solution with a large surface exposed to air. The solution is stirred, avoiding bubbles, when hydrosulphite is added. Oxygen gradually diffuses into the solution; but at a depth of 4–5 mm. material may remain reduced for 5–10 minutes if the solution is not further disturbed, or reoxidation will occur immediately on further stirring. By using only enough hydrosulphite to decrease oxygen content somewhat without actually reducing the *Patiria* material and by sealing at once, reduction by oxygen uptake may be speeded up. With relatively large amounts of hydrosulphite, but still only a small fraction of a milligram for 1 ml. of solution, reduction is almost instantaneous, with little or no evidence of regional difference.

In material sealed as soon as possible after indophenol reagents are added in standard concentrations, regions which would react most rapidly in open solution may remain completely colorless or may show less color than other parts, while regions of less rapid reaction in open solution become more deeply colored. On readmission of oxygen the normal reaction pattern appears at once. With still lower oxygen content, individuals may remain entirely without color. Under these conditions the reduction gradient may appear, or reduction may be complete without formation of oxidized indophenol at any time until the preparation is opened. Evidently, the presence of a reaction gradient is not necessary for the appearance of a reduction gradient.

With very low concentrations of reagents, regions which show more rapid reaction in higher concentrations may show less reaction than other regions or none at all, even in open solution with high oxygen content. In these cases the very small amounts of intracellular indophenol are either kept in reduced form or otherwise disposed of in the regions which show more rapid reaction in higher concentrations. Similar reversals of reaction gradients in very low concentrations of methylene blue, Janus green, and indophenol have been observed in other material (Child, 1942*a*, *b*) in the presence of high oxygen content of solution. These cases suggest the possibility that very small amounts of all these substances may be disposed of in some other way than merely by being reversibly reduced, at least in the "higher" regions of the gradients.

Reduced indophenol is evidently less

toxic than oxidized, not only for echinoderm material but for protozoans, oligochetes, and various other forms. With reduction of intracellular indophenol by oxygen uptake in sealed preparations, motile stages of *Patiria* may continue active swimming, apparently until they die from lack of oxygen—at any rate, much longer than portions of the same lot open to air. On readmission of oxygen to the reduced preparation while swimming continues oxidation takes place rapidly; and, if intracellular concentration of indophenol is relatively high, movement stops almost at once, and cytolysis may follow soon.

Since most reaction and reduction gradients coincide in direction in the same region, the question arises whether or to what extent the reduction gradient may be a result of the reaction gradient. Does oxidized indophenol stimulate or excite its own reduction, so that certain regions reduce more rapidly than others because they have reacted more rapidly and contain a higher indophenol concentration? As regards this question, it may be noted, first, that indophenol reduction gradients in *Patiria* are closely similar to reduction gradients of methylene blue and Janus green, although staining by these latter dyes preceding reduction is apparently uniform. Second, as noted above, with low oxygen from beginning of exposure to the indophenol reagents the region which would show more rapid reaction with more oxygen shows less than other regions, or none at all. That indophenol is present in the region concerned is shown by appearance of the usual gradient on admission of oxygen. Third, with continued reaction, reduction is first retarded or inhibited in regions of most rapid reaction, with progress of retardation to other regions. And, finally, reduction gradients do not always parallel reaction gradients. The most conspicuous reduction gradient in the larval development of *Patiria*, the acropetal gradient of the later blastula, is opposed in direction to the reaction gradient (p. 136). In the very minute amounts present in earlier stages of reaction, indophenol apparently irritates or excites to some extent, and may perhaps influence reducing enzyme activity; but with continued reaction it certainly retards all visible activity, while reduction gradients are still unaltered. It seems beyond question that reduction gradients, as well as reaction gradients, represent factors of physiological pattern. By use of different concentrations of reagents and with reduction at different stages of reaction, it is not difficult to distinguish the reduction patterns of essentially uninjured individuals from those resulting from toxic effects.

Reoxidation.—On readmission of oxygen to the sealed preparation after reduction, or diffusion of oxygen into the open solution after hydrosulphite, reoxidation and recoloration of intracellular indophenol occur rapidly or slowly, according to conditions. In general, the pattern of recoloration has been regarded as less significant and has received somewhat less attention than patterns of reaction and reduction because it is dependent, to some extent, on oxygen content of the solution and because, after reaction and reduction, toxic effects often appear on reoxidation. With rapid increase of oxygen content of the solution to a relatively high level, reoxidative recoloration progresses in the same direction as reaction and reduction; that is, regions of more rapid reaction and reduction show recoloration more rapidly than others. With gradual increase of oxygen content, recoloration may progress in the opposite direction, with regions of more rapid reaction and

reduction reoxidizing less rapidly than others. In other words, with sufficiently high oxygen content of solution rapidly attained, reoxidation is more rapid in regions of higher, than in those of lower, oxidase activity. The reverse progress of reoxidation with relatively low oxygen content of solution suggests that regional differences in oxygen uptake may perhaps be involved; intracellular oxygen content may become sufficient to reoxidize indophenol earlier in regions of lower than in those of higher oxygen uptake. Similar differences in the course of reoxidation with difference in oxygen content of solution have been observed in various other organisms with methylene blue and other oxidation-reduction dyes, as well as with indophenol. For example, after reduction a *Paramecium* swimming into a region of relatively high oxygen content at the surface or margin of the solution reoxidizes from the anterior end; remaining in deeper parts of the solution with less oxygen, it reoxidizes from the posterior end anteriorly. Both reoxidation patterns have been seen in the same individual with repeated reduction and reoxidation as it swam into different regions of solution into which oxygen was diffusing after reduction.

After moderate reaction, regions of more rapid reaction and reduction often show less color on reoxidation than in the primary reaction, and sometimes less color than other regions; or they may even remain colorless. In these cases no further recoloration has been observed with prolonged aeration of the solution, again suggesting that indophenol in low concentrations may gradually be disposed of in some other way than by reduction in these regions (see p. 132).

Photodynamic effects of indophenol.—Indophenol is photodynamically active, even in bright diffuse daylight to some extent; consequently, material was kept in darkness or in dim light after addition of reagents, except during periods of examination. Toxic effects and death occur earlier in light than in darkness, but special study of photodynamic effects of indophenol on the *Patiria* material has not been made.

Indophenol and developmental pattern.—With the procedures above described, indophenol provides three lines of evidence—the primary differential reaction, differential reduction, and differential reoxidation—concerning the spatial oxidation-reduction pattern in living, intact developmental stages of *Patiria*. Also, the differential alterations of pattern with very low concentrations of reagents and with toxic effects give further evidence of the presence of graded quantitative differences in physiological condition, constituting definite spatial patterns. As regards spatial relations, patterns of indophenol reduction and reoxidation are virtually identical with those of reduction and reoxidation of methylene blue and Janus green; the indophenol reaction provides a further line of evidence concerning patterns of oxidase activity.

Presentation of data.—It is, of course, quite out of the question to describe and figure, for each developmental stage, a series of stages of indophenol reaction, reduction, reoxidation, and the modifications with difference in concentration of reagents and differential toxic effects. Immobilization of motile stages for photomicrographic purposes introduces complications, and brilliant illumination on the microscope stage soon produces photodynamic effects. The attempt is made in the following text to describe and indicate diagrammatically in figures the most significant and constant features of pattern which appear, on the

basis of a comparison of effects of a wide range of concentration of reagents, periods of reaction, rapid and gradual oxygen decrease and increase, and obvious differential toxic effects, to be characteristic of uninjured developmental stages. Attention is also called to secondary or incidental modifications appearing under certain conditions. The patterns regarded as normal have been observed repeatedly in different lots, totaling hundreds of individuals. With proper procedure they appear in all individuals of a lot except in variations of asymmetry, and they can be made visible in other lots of the same stages, as desired.

In most of the diagrammatic figures, directions of progress, rather than stages of reaction and reduction, are indicated by arrows. In the general polar gradient patterns, the longer arrows indicate more rapid progress of reaction or reduction, that is, less steep gradients, than shorter arrows. In later stages, size of organ or part may limit the length of indicator arrow. Certain figures are attempts to indicate visible regional differences at certain stages of reaction or reduction, chiefly as evidence of changes during development. Differences in time of reaction in different regions or organs of the more advanced larval stages are indicated in certain figures by numerals.

The terms "apical" and "basal" are used in preference to "animal" and "vegetal" for the polar regions, and "acropetal" and "basipetal" for directions of progress of reaction or reduction in earlier stages. In later stages, apical becomes anterior; basal, posterior and finally posteroventral. Intracellular appearance of the blue color of indophenol is commonly designated "reaction"; the reversible decoloration in low oxygen, "reduction"; and recoloration with oxygen increase following reduction, "reoxidation." A gradient is basipetal or acropetal according as the color-change progresses basipetally or acropetally.

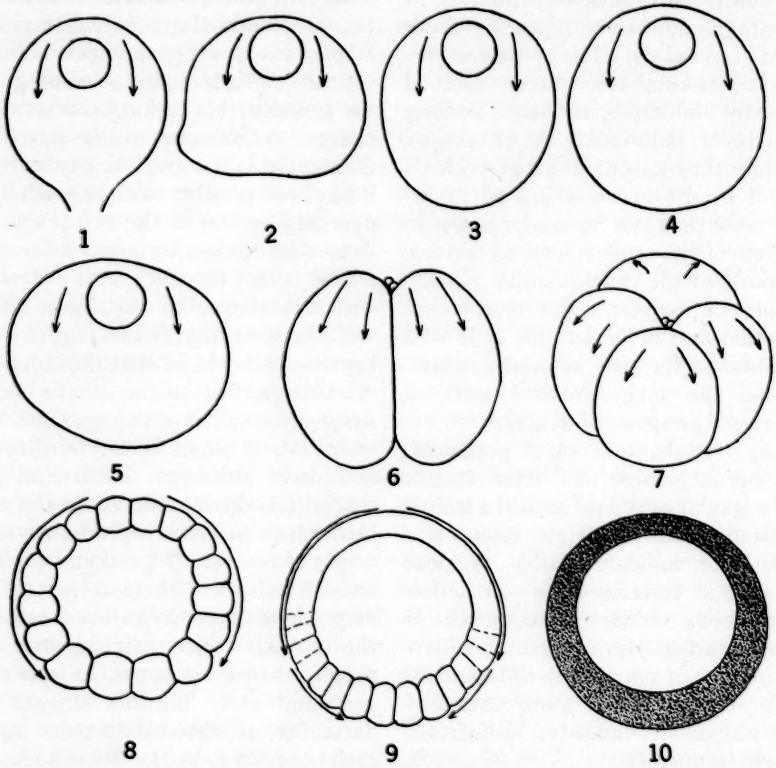
OÖCYTES, CLEAVAGE STAGES, AND EARLY BLASTULAE

In half-grown and later oöcytes from teased gonads the indophenol reaction is most rapid where the nucleus is nearest the cell surface, and with progress of the reaction this region becomes almost opaque earlier than other parts. The differential is not great at any level, and it has been possible to distinguish it only over half or less of the cell (Figs. 1–5). According to earlier observations, the region where the nucleus is nearest the surface becomes the apical pole (Wilson and Mathews, 1895; Child, 1936). Granting this, it is evident that the indophenol reaction gradient of the oöcyte becomes the polar gradient of the egg. The differential is still slight in the fertilized egg and early cleavages. In 2- and 4-cell stages it is distinguishable in the apical hemisphere or apical third in the earlier stages of reaction (Figs. 6 and 7); in the basal hemisphere there seems to be a very slight gradient. In the 8-cell stage the 4 smaller cells react a little more rapidly than the 4 larger. In later cleavages and early blastula stages, while flattening of the blastomeres against each other to form the smooth blastular epithelium is progressing basipetally, a basipetal reaction gradient becomes visible over the whole blastula but is still not steep (Figs. 8–10). The very limited time available for observations on oöcytes and early developmental stages was devoted to study of the reaction only. However, basipetal indophenol reduction has been observed in the echinoid *Dendraster* (Child, 1941*b*), and earlier data on reduction of methylene blue and Janus green show a basipetal reduction gradient.

Preceding toxic effects of indophenol, recoloration on reoxidation following reduction in cleavage and early blastula stages may progress either basipetally or acropetally, according to conditions (see p. 133). After differential retardation of reduction by the toxic effect of intracellular indophenol the apical region, in which reduction is most retarded, or completely inhibited except under extreme conditions, reoxidizes first, if reduction occurred in it. At a given level, reoxidation progresses from the external surface inward.

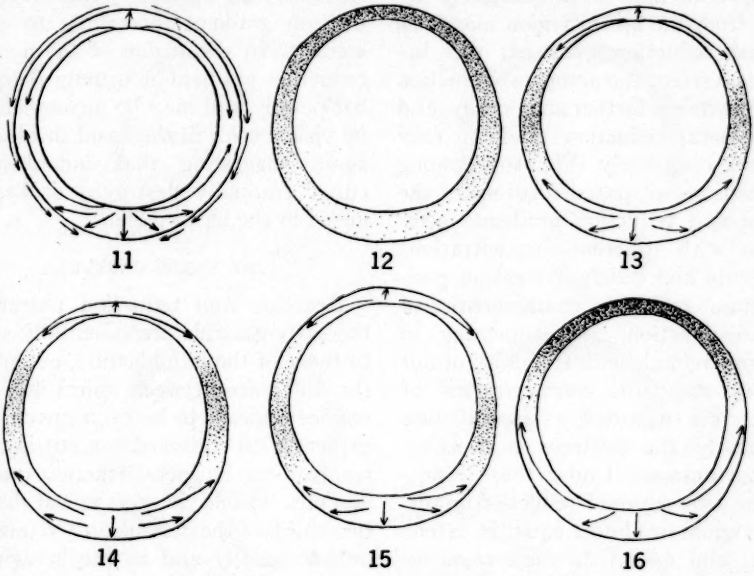
THE LATER BLASTULA

In later blastula stages a decrease in thickness of the wall progresses basipetally from the apical region, and a very striking change in certain aspects of indophenol pattern appears. Reaction progresses basipetally, as in earlier stages; but the reaction gradient is now steeper, and the reaction in the basal



FIGS. 1–10.—Reaction patterns in oöcytes, cleavage stages, and early blastula. Figs. 1–5, oöcytes of various stages, with most rapid reaction where nucleus is nearest cell surface; ovarian attachment indicated in Fig. 1. Figs. 6 and 7, reaction gradient in 2- and 4-cell stages, not certainly distinguishable in basal half. Fig. 8, early blastula, slight reaction and reduction gradients both indicated by the same arrows. Fig. 9, slightly later blastula, with basipetal flattening of blastomeres against each other to form smooth epithelium; reaction and reduction gradients basipetal, both indicated by the same arrows. Fig. 10, an attempt to indicate, by density of stippling, the slight differential in the reaction gradient of the early smooth-walled blastula.

region is relatively, and apparently absolutely, less rapid than in earlier blastula stages (Figs. 11 and 12). With standard concentrations of reagents, the apical region becomes deep blue, while there is still little or no reaction basally. With high concentrations of reagents, there is less apicobasal difference, but considerably slower appearance of intracellular indophenol. The character of this change is, of course, not known; but three possibilities are suggested: first, the basal region has become capable of reducing low concentrations of indophenol without decrease of oxygen content of the solution; second, its ability



FIGS. 11–16.—Reaction and reduction patterns in the later blastula. Fig. 11, basipetal reaction gradient indicated by arrows external to blastula wall, the two opposed basipetal and acropetal reduction gradients, by arrows in the wall, also indicating that reduction progresses from the blastocoelic surface outward at any level. Fig. 12, the reaction gradient of the later blastula. Figs. 13–15, variations in extent of the two opposed reduction gradients. Fig. 16, reduction progressing acropetally after retardation of reduction apically and reversal of the basipetal reduction gradient by toxic effect of intracellular indophenol.

still distinctly more than in earlier blastulae. With long-continued reaction, the blastula may become almost or quite uniformly deep blue; this happens only after movement has stopped, and reduction, after reaction has reached this stage, is retarded or partial.

The reaction gradient, preceding toxic effects, differs from the very slight gradient of earlier stages and indicates a rather sudden change in physiological condition in the basal region with a conto destroy indophenol in some other way has increased; third, a differential decrease in oxidase activity, greatest about the basal pole, has developed.

Reduction after reaction has progressed to the basal region shows two opposed gradients. Reduction usually becomes evident about the basal pole earlier than in any other region and progresses acropetally. A basipetal reduction gradient from the apical region also appears, and at all levels reduction

progresses from the blastocoelic surface outward (Figs. 11 and 13). The last region to reduce varies in position according to conditions. With moderate reaction it is at, or somewhat above or below, the equator (Figs. 13–15). With deeper indophenol color and presumably higher intracellular indophenol concentration, reduction is progressively retarded from the apical region along the basipetal reduction gradient; this becomes less steep, the acropetal reduction gradient extends farther acropetally, and the basipetal reduction gradient may disappear completely (Fig. 16). Among the variations in spatial relations of the two opposed reduction gradients after reaction with different concentrations of reagents and different reaction periods, those relations characteristic of moderate reaction, and appearing in swimming animals with standard, or not far from standard, concentrations of reagents, are regarded as representing most nearly the patterns of normal, uninjured animals. Under these conditions the two opposed reduction gradients are equal or almost equal in extent (Figs. 11 and 13–15). In these cases reduction is almost always evident in the basal region earlier than apically. However, in view of the possibility that the differential reaction preceding reduction may have altered physiological condition differentially, some degree of uncertainty as regards the completely normal pattern perhaps remains. But, whatever the normal relations of the two gradients, the change in physiological condition in the basal region from earlier stages is evident.

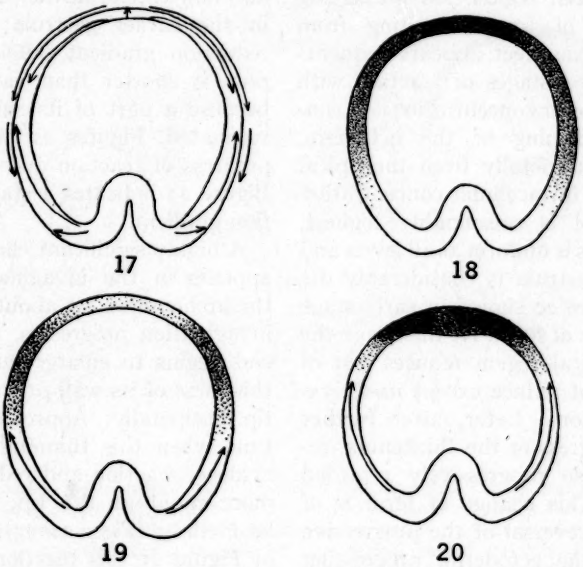
Basipetal reaction and reduction in the later blastulae appear to progress a little more rapidly on one side, presumably the ventral. A similar difference in dye reduction on the two sides of the later blastulae was noted and figured in an earlier paper. Dorsal and ventral sides are not morphologically distinguishable in these blastulae. As far as observed, the acropetal reduction gradient does not show such difference.

Recoloration in reoxidation of reduced indophenol after moderate reaction may progress basipetally or acropetally in the region of the basipetal reaction and reduction gradient, according to oxygen content. In the region of the acropetal reduction gradient it usually progresses basipetally and may be incomplete with no visible color in the basal third or less, again suggesting that indophenol is either gradually destroyed or kept reduced in the basal region.

THE EARLY GASTRULA

Reaction and reduction patterns in the early gastrula are essentially similar to those of the late blastula, except that the difference between apical and basal regions appears to be even greater than earlier. With standard concentrations of reagents the basipetal reaction gradient becomes visible in the apical half to two-thirds of the gastrula in 5–6 minutes; farther basally and in the invaginated entoderm, there is no appreciable reaction until much later. The external arrows in Figure 17 indicate the ectodermal reaction gradient, and Figure 18 is an attempt to indicate the visible differential. With continued exposure to the reagents, the reaction slowly progresses basipetally, and after about an hour is usually visible as far as the blastopore; the invaginated entoderm is still usually without reaction or with only a trace; the apical ectoderm is very deeply colored. Evidence from somewhat later stages indicates that absence of reaction in the invaginated entoderm is due not to failure of the reagents to reach it but to its physiological condition. With higher concentrations, reac-

tion is, of course, more rapid and may extend to the invaginated entoderm. Under these conditions the apical ectoderm becomes almost opaque with indophenol, while there is still only slight color basally. This very distinct gradation from deep blue apically to almost or quite colorless basally in swimming animals is a striking and beautiful visible expression of certain factors of physiological pattern. With decreasing concentrations the basipetal reaction gradient remains distinct in the apical two-thirds or half, but there is less progress of the reaction farther basipetally until the basal third to half and the invaginated entoderm remain entirely without appreciable reaction. Here, as in the later blastula, the basal region either reduces indophenol or destroys it in some way. Reduction, after reaction has become visible in the basal region, shows two opposed gradients like the later blastula (Figs. 17 and 19). Basipetal reduction from the apical region becomes evident somewhat later than acropetal and progresses over the apical half more or less until reduction is retarded by toxicity of indophenol. With high concentrations or long-continued reaction, reduction is retarded apically, and the acropetal gradient extends farther, until the apical ectoderm reduces last or fails to reduce. Reaction in the invaginated entoderm is so slight, under the usual conditions, that presence of a gradient pattern is uncertain at this stage. With concentrations considerably above standard and a long reaction period, a slight gradient from the tip toward the blastopore seems to be present in some individuals. Comparison with the



FIGS. 17–20.—Reaction and reduction patterns in the early gastrula. Fig. 17, basipetal reaction gradient indicated by arrows external to gastrula wall; the two opposed reduction gradients, by arrows in the wall. Fig. 18, the reaction gradient. Fig. 19, the two opposed reduction gradients. Fig. 20, a stage in basipetal thickening of the wall; retardation or inhibition of apical reduction by toxic effect of intracellular indophenol.

later gastrula suggests that this gradient is just developing. Reduction of methylene blue and Janus green apparently decreases slightly for a short distance in both directions from the region of invagination (Child, 1936). With indophenol this gradient has not been observed with certainty, probably because of slight reaction and rapid reduction in the whole basal region. An interesting modification of form, resulting from differential toxic effect, appears frequently in advanced stages of reaction with standard or lower concentrations. It consists of thickening of the ectoderm, progressing basipetally from the apical region, where intracellular concentration of indophenol is presumably highest, until thickness is uniform at all levels and size of the gastrula is considerably decreased. Figure 20 shows an early stage in this change of form. At this stage the thickened apical region reduces last of all or does not reduce except under extreme conditions. Later, after further basipetal progress of the thickening, reduction is also progressively retarded basipetally. This change of form is of interest as a reversal of the progressive thinning of the ectoderm, progressing from the apical region basipetally, in the later blastula and the gastrula.

Reoxidative recoloration following reduction is more rapid in the apical than in the basal hemisphere; and more or less of the basal region and the invaginated entoderm usually remain almost or entirely without color, even though there was some reaction, at least in the external entoderm. Again it appears that the basal region either keeps indophenol reduced or disposes of it in some way.

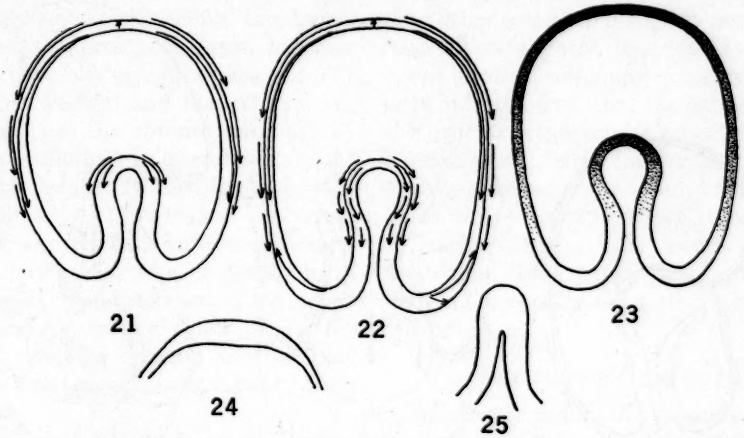
THE LATER GASTRULA

Reaction and reduction progress basipetally in the ectoderm over about three-fourths of the length and apparently a little more rapidly on one side, probably the ventral side. The basal region reacts so much less rapidly that the color gradient is brilliantly distinct in the swimming gastrula; but the basal, almost or quite colorless, region is less extensive than in the earlier gastrula. Basipetal reaction and reduction gradients represent essentially the ectoderm and now extend farther basipetally than in the earlier gastrula; the acropetal reduction gradient outside the blastopore is shorter than earlier, evidently because a part of its substrate has invaginated. Figures 21 and 22 indicate progress of reaction and reduction, and Figure 23 indicates a stage of the reaction gradient.

A highly significant change in pattern appears in the invaginated entoderm, the archenteron, at about this stage. As invagination progresses, the apical free end begins to enlarge; and decrease in thickness of its wall progresses from the tip basipetally. Approximately at the time when this thinning first becomes evident, reaction and reduction become more rapid at the tip, with decrease basipetally (Figs. 21–23). At the stage of Figure 21 this reaction becomes visible later than in the apical ectoderm, and the gradient is relatively slight and becomes indistinct or absent in the thick-walled entoderm. At the later stage of Figures 22 and 23, when apical thinning has progressed farther, reaction is relatively earlier, almost or quite as early as in the apical ectoderm, and both reaction and reduction gradient extend farther and have become distinctly steeper. Relative times of appearance and rates of reaction and reduction probably do not indicate exactly differences in condition in apical ectoderm and archenteron tip. Presumably, penetration of reagents into the blastocoel, though certainly rapid, is not in-

stantaneous. Also, the fact that, in general, reduction at any level progresses from the blastocoel outward indicates that oxygen content is considerably lower in the blastocoel than outside, as might be expected. Methylene blue and Janus green reduction also show reduction from the blastocoel outward (Child, 1936). Consequently, archenteric reaction may be somewhat later, and occur after more or less reaction, they appear more deeply colored than corresponding unaltered regions of other individuals of the same lot. This difference is apparently due to thickness of the cell mass, not to more rapid reaction; the thickened regions reduce very slowly, or not at all, under the usual conditions.

With reaction sufficient to show the entodermal gradient clearly, reoxidation



FIGS. 21–25.—Reaction and reduction patterns in later gastrula stages Fig. 21, stage of beginning enlargement of free end of archenteron, with basipetal ectodermal reaction gradient (arrows outside wall) and reduction gradient (arrows in wall) and new reaction and reduction gradient appearing in enlarging tip of archenteron. Fig. 22, a somewhat later gastrula, with ectodermal reaction and reduction gradients as in Fig. 21 and a short acropetal reduction gradient in the basal region which appears when reduction follows a reaction period sufficient for visible reaction basally. Fig. 23, the reaction gradient. Fig. 24, apical region of a gastrula, showing a stage of basipetal thickening of the wall by the toxic effect of indophenol. Fig. 25, loss of epithelial character and aggregation of cells of enlarged tip of archenteron by toxic effect.

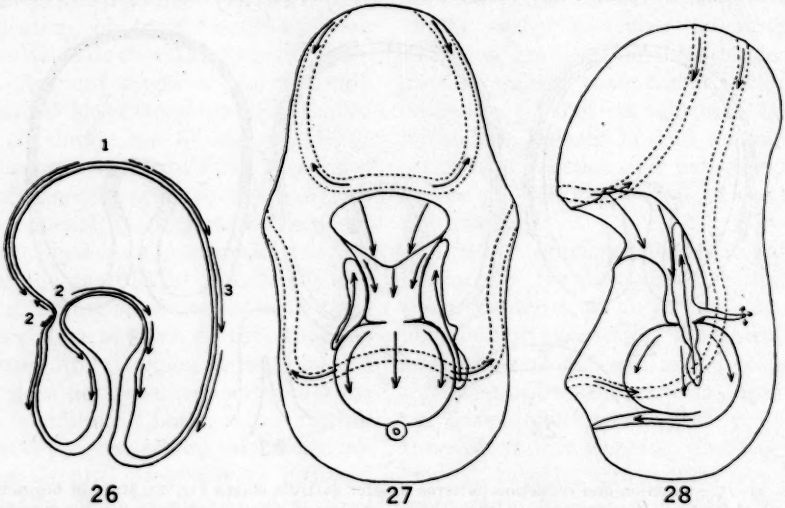
reduction somewhat earlier, than its physiological condition alone would determine.

Thickening of ectoderm, progressing from the apical region, with decrease in size of the gastrula (Fig. 24) and a reversal of the enlargement and thinning of the apical archenteron, often progressing until it becomes a solid cell mass but without cytolysis and apparently still living (Fig. 25), appear frequently with gradually developing toxicity of indophenol. Since these cell aggregations after reduction usually progresses acropetally in both ectoderm and enlarged archenteric tip, except that the apical ectoderm is usually injured and reoxidizes early, instead of late. With less reaction and with high oxygen content, ectodermal reoxidation may be basipetal.

ECTODERM, STOMODEUM, AND CILIATED BANDS OF LATER STAGES

At the stage of early stomodeal invagination, ectodermal reaction pro-

gresses posteriorly and slightly more rapidly ventrally than dorsally but with some local alterations. A zone of slightly more rapid reaction has been observed in some larvae posterior to the ventral concavity, but not extending to the blastopore. Since this zone has not been seen in all individuals and apparently is not associated with any distinguishable earlier than adjoining ectoderm anterior to it. This region is the only part of the rapidly reducing basal half, more or less, of the late blastula which has not invaginated. Ectodermal reoxidation may progress posteriorly or anteriorly, according to conditions: if thickened anterior ectoderm reduces, it reoxidizes first.



FIGS. 26–28.—Fig. 26, stage of stomodeal invagination; ectodermal reaction and reduction both basipetal, except for stomodeal invagination, and both indicated by the same arrows, ventral side slightly in advance of dorsal; reaction and reduction most rapid in anteroventral region of archenteron; relative times of reaction and reduction in various regions indicated by numerals *1*, *2*, *3*. Figs. 27 and 28, advanced larval stages from ventral and left side.

developmental activity in this region, its significance remains uncertain. Reaction is slightly more rapid in the early stomodeal invagination than in adjoining ectoderm (Fig. 26). Ectodermal thickening, progressing from the extreme anterior region over half or more of the preoral body, with corresponding decrease in size, is a frequent toxic effect.

The normal reduction pattern is, in its general characteristics, similar to the reaction pattern, except that a region about the blastopore, provided it shows appreciable reaction, apparently reduces

In the most advanced, gradually starving larval stages (Figs. 27 and 28) the ectodermal reaction pattern with standard or lower concentrations is often more or less completely reversed in open, aerated solution, with the anterior and ventral regions showing less reaction than posterior and dorsal. Since this reversal appears with the lower concentrations of reagents, it suggests destruction of indophenol. With concentrations three or four times the standard, reaction and reduction progress posteriorly and dorsally, as in earlier stages; and

reoxidation progresses anteriorly and ventrally in all cases observed, except that the anterior ectoderm, when injured by indophenol, reduces late and reoxidizes early.

Large numbers of larvae have been used in many lots with a wide range of concentrations of reagents and different relative concentrations of the two in the attempt to determine reaction and reduction of the ciliated bands in relation to adjoining ectoderm. As the bands differentiate, their ectoderm becomes thicker than the general ectoderm. The transverse ventral and the extreme anterior parts of the anterior band are almost twice as thick as lateral parts in the advanced larvae. In absence of further development, it remains uncertain whether the regional differences represent differences in rate of development or are final. Differentiation of the posterior band progresses chiefly from the ventral side; its extreme anterior part appears to be slightly in advance of lateral parts.

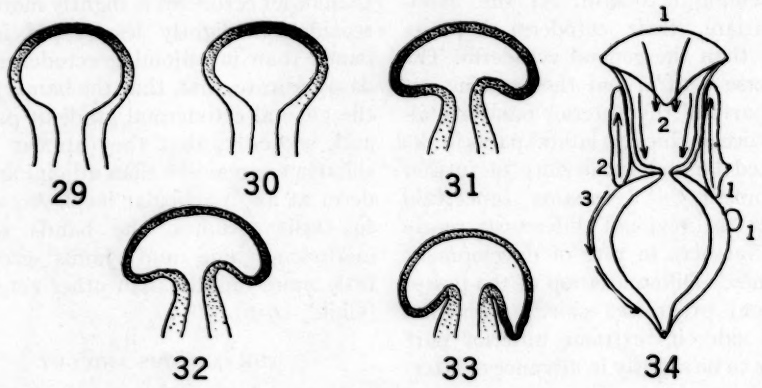
With standard or lower concentrations of reagents, either the bands in earlier stages of reaction are not distinguishable from other ectoderm or ventral and anterior parts react a little less rapidly than does the adjoining ectoderm. It was noted above that under these conditions the extreme anterior region, where the two bands lie close together, reacts relatively slowly. Also, reaction is usually slightly more rapid ventrally in the posterior than in the anterior band. During these earlier reaction stages the larvae swim normally. With further progress of reaction the bands become distinctly deeper in color than other ectoderm; and, as this takes place, swimming becomes slower and very soon stops. With concentrations three or four times the standard, the bands react more rapidly from the earliest stages of reaction than does general ectoderm, and more rapidly ventrally and anteriorly than elsewhere; movement is retarded as the reaction becomes visible. The relatively slow reaction with low concentrations suggests that ability to reduce or destroy indophenol in low concentrations is slightly greater in the bands than in other ectoderm. Reduction in low oxygen after slight reaction in the bands and before they become deeper than other ectoderm is slightly more, and reoxidation slightly less, rapid in the bands than in adjoining ectoderm. The data indicate, first, that the bands retain the general ectodermal gradient pattern and, secondly, that they appear to be slightly more active than adjoining ectoderm at any particular level. According to earlier studies, the bands reduce methylene blue and Janus green a little more rapidly than other ectoderm (Child, 1936).

THE COELOMS AND GUT

As already noted, the expanded, thin-walled tip of the archenteron develops a much more rapid reaction and reduction than the thick-walled part posterior to it. As long as the expanded region remains almost spherical, no definite and consistent dorsiventral or lateral differences in reaction and reduction gradients from the median anterior region have been observed in the majority of a large number of individuals (Fig. 29). Not infrequently, however, the region of most rapid reaction and reduction appears to be slightly to the left or to extend slightly farther to the left than to the right of the median region (Fig. 30, from the ventral side). These cases suggest the possibility that this asymmetrical reaction and reduction represent an early stage in development of larval asymmetry, preceding its morphological appearance in coelom development, and that the indi-

viduals showing it are slightly in advance of others or have a more pronounced asymmetry.

With further development the expanded region of the archenteron extends ventrally toward the stomodeal invagination. As this change in shape takes place, the region of most rapid reaction and reduction, as seen from the side, becomes anteroventral (Fig. 26). As the lateral coelomic extensions of the thin-walled archenteric region develop, reaction and reduction become slightly more rapid at their tips than elsewhere. Here, again, individuals apparently differ. In the majority of individuals no definite difference in reaction and reduction gradients on the two sides has been observed (Fig. 31, from ventral side), but in many individuals the left side appears to react and reduce a little more rapidly than the right (Fig. 32, from ventral side). Very rarely, a case was seen with the right side apparently in advance. Since the left side is almost always in advance, it seems probable that the difference does represent developing asymmetry. Later stages show occasional reversal of morphological asymmetry, and the rare cases with the right side apparently in advance may be early stages of such reversals. As the coelomic diverticula elongate posteriorly, they show distinct gradient patterns, with reaction and reduction most rapid at the posterior ends; and the left usually reacts and reduces a little more rapidly than the right, more distinctly so when it is longer (Fig. 33, from ventral side). With further elongation these gradients persist and appear even more distinct, with the left side almost always in advance. A few cases of morphological reversal of asymmetry, with the madreporic canal developing from the right instead of the left coelom, showed slightly more rapid reaction and reduction on the right. The posterior coelomic bud, usually developing somewhat dorsally on the left side of the midgut, reacts and reduces about as rapidly as the posterior region of the anterior coelom (Fig. 34, from ventral side), with which it later unites.



FIGS. 29–34.—Gradient patterns in coelom development from ventral side. Fig. 29, enlarged tip of archenteron with gradient from median region. Fig. 30, most rapid reaction slightly to the left. Fig. 31, reaction gradients apparently equal from tips of coelomic diverticula. Fig. 32, reaction slightly more rapid at tip of left coelom. Fig. 33, posterior elongation of coeloms beginning, left side in advance and with more rapid reaction than right. Fig. 34, later stage of coeloms, stomodeum, and gut; reaction and reduction gradients indicated by the same arrows, time order by numerals.

In the most advanced larvae observed,

reaction and reduction gradients from the posterior regions of the two coeloms persist, with the left in advance (Figs. 27 and 28), except in one case of reversal. As the madreporic canal develops, it seems to show slightly more rapid reaction and reduction than does the coelomic region from which it arises. As it reaches the ectoderm of the body wall, a small area, apparently temporary, of more rapid reaction and reduction appears about the pore (Fig. 28).

The rapid penetration of the reagents into the blastocoel is indicated by appearance of the reaction posteriorly in the coeloms almost or quite as soon as in the anterior ectoderm. If they were directly exposed to the reagents, reaction would probably be even more rapid than in the ectoderm. With hydrosulphite, coelomic reduction is as rapid as ectodermal; with reduction in sealed preparations, it is usually more rapid than ectodermal, presumably because the oxygen content is lower in the blastocoel than outside. As in other gradients, coelomic reduction is sooner or later differentially retarded by continued reaction with reversal or obliteration of the gradient or incomplete reduction. The reoxidation gradient is opposed in direction to reaction and reduction gradients, at least usually. High oxygen content is probably not attained rapidly enough in the blastocoel to permit reoxidation in the same direction as reaction and reduction. However, after moderate reaction and reduction the color on reoxidation finally becomes deeper posteriorly in the coeloms, as in the primary reaction. With rapid and thorough aeration after reduction, the deeper color appears posteriorly so early that direction of progress of reoxidation becomes uncertain.

With development of fore-, mid-, and hindgut from the entoderm, reaction and reduction become more rapid and still progress, in general, from anterior to posterior, but with some modifications. In the thin-walled stomodeum and foregut or oesophagus rate of reaction and reduction decreases from the mouth to the midgut and from the much longer anterodorsal to the shorter posteroventral side (Figs. 27 and 28). The anterodorsal side reacts and reduces about as rapidly as the coeloms. In the thick-walled midgut, reaction and reduction progress posteriorly and, so far as observed, equally along all meridians (Figs. 27 and 28). The anterior region of the midgut usually shows reaction and reduction earlier than the posteroventral wall of the foregut. From the midgut, reaction and reduction progress posteriorly along the hindgut. Immediately about the anus a region of more rapid reaction has been seen in some advanced larvae, but its possible relations to development or function have not been investigated. Progress of reoxidation is opposed in direction to reaction and reduction gradients, so far as observed—again probably because high oxygen content is not attained rapidly enough in the blastocoel to permit reoxidation in the same direction as reaction and reduction.

DISCUSSION

Definite patterns of indophenol reaction and reduction in larval development of *Patiria* appear in all individuals under properly controlled conditions, undergo definite changes in the course of development, and are very evidently associated with developmental activities. Reoxidation patterns apparently depend to some extent on oxygen content of the medium in relation to oxygen use in different regions; they are evidently correlated with reaction and reduction patterns. All three features of pattern are subject to alteration by toxic effects of intracellular indophenol or, in some

cases, of the reagents, and may also show alteration with very low concentrations of reagents, probably because indophenol in very low concentrations may be destroyed, at least by certain regions. All these alterations of pattern, however, are also differential and definite and constitute further evidence of presence of a spatial physiological pattern.

In general, indophenol reduction pattern is essentially similar to the reduction pattern observed with methylene blue and Janus green (Child, 1936); but certain features—for example, the gradient patterns of the coeloms, which remained uncertain or could not be distinguished with those dyes—have appeared clearly with indophenol. The relatively intense color of exceedingly minute amounts of intracellular indophenol, as compared with the dyes used earlier, is at least in part responsible for the greater distinctness of regional differences. Moreover, indophenol brings to light a feature of physiological pattern involving activity of an oxidase, now regarded as cytochrome oxidase, which is not indicated by reduction in which the flavoprotein-dehydrogenase system is concerned.

All spatial patterns indicated by indophenol reaction and reduction, as well as by reduction of methylene blue and Janus green, are gradients, except the sharp delimitation of the ciliated bands of later larval stages from general ectoderm, evidently an expression of differentiation of the bands. Within the bands there is gradient pattern. The regions of most rapid reaction and reduction, the "high" regions of the gradients, are definitely localized; but the "low" ends are more or less indefinite in position, except when the gradient is coextensive with the individual body. Since the gradients are expressions of activities differing in rate but occurring in all parts, this indefiniteness in extent is to be expected. The extent of a particular gradient may depend on its relation to other gradients, and these may differ with developmental stage and may be altered by experimental procedure.

Two relatively sudden and conspicuous changes in gradient pattern appear during earlier developmental stages. The first of these, the appearance in the later blastula of a high rate of reduction in the basal region, with graded decrease acropetally, apparently indicates a very considerable and rather sudden change in physiological condition in the region representing, at least approximately, the prospective entoderm. In the early blastula, no trace of this gradient has been observed; reduction, like reaction, progresses basipetally. The extent of the acropetal gradient varies with experimental conditions. Preceding toxic effects, it meets the basipetal reduction gradient somewhere near the equator. However, if reduction is differentially retarded toward the apical pole, acropetal reduction may extend into the apical hemisphere, or sometimes even to the apical pole, which then becomes the low end of the gradient because reduction is more retarded in it than in any other region. In the earlier paper on reduction, attention was called to the greater thickness basally of the blastula wall as perhaps playing some part in determining or exaggerating the acropetal reduction gradient (Child, 1936). However, it is evident from indophenol reaction, reduction, and reoxidation that the acropetal reduction gradient does represent a real and rather sudden change in condition. There is no sudden increase in thickness basally at the time the acropetal gradient appears; the actual cell volume is little, if any, greater than earlier.

The appearance basally of this new reduction gradient, opposed in direction

to the reaction gradient, suggests the possibility that a qualitative, as well as a quantitative, change in condition may be taking place at this time in the prospective entodermal region, with reduction, rather than oxidase activity, as one of its predominant characteristics. Whether or not this is the case, the relation of the acropetal reduction gradient to the "vegetative gradient," one of the two specifically different gradients postulated by Runnström and his co-workers and discussed in many papers,[1] requires consideration. These two gradients, the animal and the vegetal, are assumed to be present from the beginning of development and to overlap, with the animal gradient decreasing basipetally, the vegetal acropetally. They were originally regarded as gradients of concentration of specifically different substances, but more recently they have been discussed in terms of different metabolisms. The acropetal reduction gradient, however, does not agree fully with the conclusions reached concerning the postulated vegetal gradient. There is no evidence that the acropetal reduction gradient is present at the beginning of development. However, there does not seem to be any conclusive evidence of the presence of the Runnström vegetal gradient at the beginning of development. The acropetal reduction gradient does extend into the region of the basipetal reaction gradient to a varying degree, according to conditions. However, it obliterates and replaces, rather than overlaps, the basipetal reaction gradient. Also, there is a basipetal reduction gradient which has the same characteristics, so far as indophenol, methylene blue, and Janus green are concerned, as the acropetal gradient. The reaction and reduction gradients, of course, give no information concerning regional qualitative differences; they show only that formation and reduction of indophenol occur at all levels and that the differences in these respects are quantitative rather than qualitative. It seems at least possible that qualitative differences between apical and basal regions are slight or virtually absent at the beginning of development, that the pattern is predominantly quantitative, and that the appearance of the acropetal reduction gradient in the later blastula may be associated with progressive development of a chemodifferentiation of the prospective entoderm. In other words, a specific vegetal gradient may itself develop instead of being preformed. However, quantitative oxidation-reduction differences are greater than at the beginning of development; this suggests that quantitative factors may still play fundamental parts in further development. The morphological developmental modifications, even the "animalized" and "vegetativized" forms, are more simply accounted for on a basis of quantitative axial differences than by the various assumptions required on the basis of the specifically different, overlapping gradients postulated by the Runnström group.

The second conspicuous change in gradient pattern also concerns the entoderm. It is the appearance of a region of rapid reaction and reduction with a new gradient at the inner free end of the archenteron as this begins to enlarge and decrease in thickness. The close association of this new gradient pattern with the change in form of this region indicates its relation to developmental activity. The later changes in reaction and reduction in this region, associated with appearance and development of the coeloms, also indicate association of indo-

[1] See Child, 1941*a*, pp. 129–43, 241–46, 436–46, and references given there; Lindahl, 1942*a*, a polemic; Lindahl, 1942*b*; Lindahl and Holter, 1940; Needham, 1942, pp. 476–502.

phenol reaction and reduction with developmental activities, in this case, with development of asymmetry.

The data on oxidation-reduction raise a further question. According to the interesting experiments of Hörstadius (1935 and other papers), the sea-urchin micromeres, which appear at the basal pole in the 16-cell stage, act as organizers of more or less vegetal parts when implanted in other regions of the embryo. The indophenol reaction and reduction of methylene blue, Janus green, and indophenol in echinoids do not indicate any marked difference between micromeres and their sister-cells, the macromeres, at the time of their formation. Later, however, the acropetal reduction gradient appears, as in the starfish; and the micromeres are at the high end of this gradient. The starfish has no micromeres and forms no mesenchyme preceding gastrulation, as does the echinoid; but development of the gastrula from the blastula is essentially like that of the sea urchin. Will cells from the basal region of *Patiria* or other species of starfish act as organizers, as do the sea-urchin micromeres? If they do, a specific organizing action of the echinoid micromeres appears somewhat doubtful. If they do not, how is the similarity of development, exogastrulation, and other modifications in starfish and sea urchin to be accounted for? These questions are still to be answered.

It has been suggested in earlier papers that the organizing action of the micromeres may depend largely or wholly on the quantitative change in condition indicated by appearance of the acropetal reduction gradient, rather than on a specific difference from other cells (Child, 1936; 1940; 1941*a*, pp. 436–46). *Patiria* development without micromeres seems to support this suggestion. Moreover, the inducing effect of the micromeres is not always the same; they do not always induce entoderm but merely make the region of implantation more basal morphologically than it would have been. It is well known that in *Patiria* entodermal invaginations, even in preoral regions, may be induced experimentally by external factors; and in the sea urchin, cells other than micromeres from the basal region may induce invagination. A point of fundamental importance, ignored by many students of developmental physiology, is that various purely quantitative external gradients, electric, photic, oxygen, pH, and temperature gradients, are of greater primary significance as organizers than the sea-urchin micromeres or the dorsal lip of the amphibian gastrula. They determine polarity and, so, indirectly the whole course of polar development. Ovarian differentials, apparently determining polarity, are probably quantitative. The organizing action of purely quantitative external differentials in determining the first step in organization suggests that specific constitution may not be necessary for organizing action by embryonic parts.

Lindahl (1942*a*, *b*) has recently maintained that the basipetal dye-reduction gradient of early sea-urchin stages cannot be "valid," because Lindahl and Holter (1940) have shown, by the Cartesian diver micromethod, that oxygen uptake is the same in apical (animal) and basal (vegetal) halves of various developmental stages of the sea urchin *Paracentrotus lividus*. In view of this conclusion, it becomes necessary to call attention to certain points concerning the apparent lack of agreement in the two lines of investigation.

First, it is not, and has not been, maintained that the dye-reduction gradients of earlier work and the indophenol reaction and reduction gradients necessarily coincide in all cases with respira-

tory gradients. Since there is no doubt of the existence and "validity" of the directly visible reaction and reduction gradients in living intact developmental stages and since their association with development appears obvious beyond all question, it seems highly probable that neither determinations of oxygen uptake or CO_2 production nor the visible reaction and reduction gradients tell the whole story as regards metabolic developmental pattern in these forms.

Second, reaction and reduction gradients in cleavage stages and early blastulae of the echinoids investigated are very slight (Child, 1936, 1941*b*); in *Patiria* they are somewhat steeper, but the difference, even between apical and basal poles, is not great. With the low concentrations of the dyes used in the earlier work on reduction and the low concentrations of the indophenol reagents found most satisfactory, the amounts of intracellular indicators required to make reaction or reduction visible are evidently exceedingly minute, and the regional differences in amounts in a slight gradient must be even less. There can be no doubt that the physiological differences made visible in such gradients are extremely small. Even if these differences are paralleled by correspondingly slight differences in respiration, it is highly improbable that such differences are sufficiently large to appear consistently and significantly with any method at present available for determination of oxygen uptake in isolated halves or other pieces of embryos. Moreover, in the later blastula and gastrula two opposed reduction gradients are present. If they parallel respiratory gradients, differences in oxygen uptake in apical and basal halves must be still less than in earlier stages. But whether oxidation-reduction gradients and respiratory gradients are always parallel or not, it appears beyond question that oxidation-reduction indicators, properly used, provide a method of great delicacy for making directly visible, uniformly and consistently in single intact living individuals in any desired number, very slight quantitative differences in physiological condition which constitute definite and characteristic features of developmental pattern. The very considerable range of variation in different determinations by Lindahl and Holter—the authors admit 10 per cent—and the necessity of calculating Q_{O_2} (= mm.^{3} $\times 10^{-4}$ per single half or whole per hour) from the total for a number of individuals indicate that even the micromethod used by these investigators is far less adequate than oxidation-reduction indicators as a means of demonstrating presence or absence of very slight regional physiological differences in echinoderm embryos.

Third, the question whether oxygen uptake is altered by the procedure involved in isolating apical and basal halves of sea-urchin developmental stages is difficult to answer with certainty. According to Lindahl and Holter, the calculated Q_{O_2} of single wholes is 99.4 ± 1.5 per cent of the two halves together. However, an error in one of the tables from which the data are taken makes this value incorrect. It is at least open to question whether any method of determining oxygen uptake at present available is adequate to give significant data on the presence or absence of slight, and perhaps temporary and individually variable, respiratory differences in entities so small as half, or even whole, sea-urchin embryos.

Fourth, indophenol reoxidation gradients suggest, though they do not prove, that regional differences in oxygen uptake, corresponding to reaction and reduction gradients, may be present and

influence the course of reoxidation under certain conditions.

Fifth, whatever the relation between oxidation-reduction gradients and respiration, the uniformity with which these gradients appear in all individuals, their very evident relation to developmental activities, and their definite changes at certain developmental stages when new developmental activities are initiated leave no doubt concerning their reality as characteristic features of developmental pattern.

Finding no differences in oxygen uptake in different isolated parts of the chick embryo, Philips (1942) concludes that the regional differences in dye reduction observed by Rulon (1935) and J. A. Miller (1941) are due merely to differences in thickness of tissue, the thicker regions reducing more rapidly because of greater tissue volume. It can readily be shown that, while differences in thickness of tissue may play some part in determining rate of reduction in some cases in the chick, they are not the only, or the chief, factor. Moreover, unpublished observations show regional differences in the indophenol reaction essentially similar, in general, to the reduction pattern, though various details of region and stage have not been worked out. Thickness of tissues is certainly not the determining factor in reduction differences in echinoderms. In the early blastula the somewhat thicker basal region reduces less rapidly than the thinner apical region; in the later blastula the thinnest apical and the thickest basal part of the wall reduce at about the same time. In the gastrula the apical ectodermal region reduces more rapidly than other parts of the ectoderm, though there is little difference in thickness; also, the enlarging tip of the archenteron reduces more rapidly than other, thicker parts of the entoderm. The posterior tips of the coeloms do not appear to be any thicker than other parts, but they reduce more rapidly. In later stages the thin-walled stomodeum and foregut reduce more rapidly than the thick-walled midgut, and the thin-walled coeloms also reduce more rapidly than the midgut. Evidently, thickness of tissue is not a very important factor in differences in rate of reduction in the echinoderm material. Again it may be questioned whether any method of determining oxygen uptake is adequate for determining the presence or absence of regional respiratory differences corresponding to the regional oxidation-reduction differences made directly visible by the indicators in the chick or in other embryonic material.

SUMMARY

1. With the use of very low concentrations of reagents, intracellular formation, reduction, and reoxidation of indophenol render directly visible certain quantitative features of pattern in living intact developmental stages of the starfish *Patiria miniata*.

2. In later stages of oöcyte growth, indophenol reaction and reduction decrease in rate from the region where the nucleus is nearest the cell surface.

3. In cleavage stages and early blastula there is a slight decrease in rate of reaction and reduction from the apical polar region basipetally.

4. In the later blastula a basipetal reaction gradient is still present, and a new reduction gradient extends acropetally from the basal region, meeting the basipetal reduction gradient in or near the equatorial region, according to experimental conditions.

5. In the gastrula a basipetal reaction and reduction gradient persists in the ectoderm, with the ventral side slightly in advance of the dorsal. As enlarge-

ment and thinning of the wall begin at the tip of the archenteron, a new gradient of rapid reaction and reduction appears there.

6. In later stages the general ectodermal pattern is still present, except for slightly more rapid reaction and reduction in the stomodeal invagination than in the ectoderm about it. As the coeloms develop, they become regions of more rapid reaction and reduction than the foregut, and the left side is often visible slightly in advance of the right. As they elongate posteriorly, rates of reaction and reduction decrease from their posterior ends anteriorly, with the left coelom often, the right rarely, in advance.

7. Reaction and reduction progress, in general, posteriorly in the gut; the anterodorsal side of the foregut reacts and reduces more rapidly than the posteroventral.

8. With gradual increase of oxygen in the medium following reduction, the reoxidation gradients are usually opposite in direction to reaction and reduction gradients or to the reduction gradient of the same region; with rapid and great oxygen increase, reoxidation may progress in the same direction as reduction.

9. Use of a wide range of concentrations of reagents and reaction periods indicates that these gradient patterns represent real physiological patterns of essentially uninjured developmental stages. They may be altered, reversed in direction, or obliterated by differential toxic effects of intracellular indophenol or high concentrations of reagents. In extremely low concentration of reagents, reaction gradients may be reversed in direction or become only partially visible, because regions which show more rapid reaction in higher concentrations apparently dispose, in some way, of indophenol in very low concentrations.

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THE CONTROL OF RECONSTITUTIONAL DEVELOPMENT IN DUGESIA DOROTOCEPHALA WITH ETHYL ALCOHOL[1]

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THE gradient system in planarians has been proved to be an important correlating mechanism, not only in the maintenance of the individuality of the organism, but in the reconstitution of pieces into whole or partial forms. Experimental or natural conditions affecting the steepness of the gradient in intact animals also affect the type of reconstitution in pieces from these animals through the inhibition or blocking of correlative factors which would otherwise be present in the pieces.[2] Gradients present in unfertilized and developing echinoderm eggs and the patterns in embryological development bear close resemblance to those underlying reconstitutional development of planarian pieces.

The present paper deals with additional experiments on the modification of gradient patterns in planarians. Experiments on animals treated with an effective agent both before and after section demonstrate further similarities between reconstitutional and embryological processes.

THE CONTROL OF HEAD FREQUENCY IN PLANARIANS

Through a large number of experiments Child and others have shown that it is possible to increase or decrease the head frequency of short transverse pieces of planarians by allowing the pieces to reconstitute under certain experimental conditions. Such agents as KCN (Child, 1916) in proper concentration will increase the head frequency of short posterior pieces and decrease the head frequency of short or long anterior pieces. The data indicate that two antagonistic actions are concerned in determining head frequency. The first of these has to do with the gradient in rate of head formation at different levels. Heads regenerate more rapidly at anterior than at posterior levels of the first zooid (Watanabe, 1935*a*), although all levels will develop a normal head if no posterior section is made. On the other hand, in short pieces particularly from posterior levels of the zooid, a head-inhibiting factor, originating at the site of posterior section, results in various degrees of differential inhibition of head development, constituting a graded series from normal to headless, with mediolateral differential most conspicuous.[3] Strong evidence has supported the view that the posterior cut surface exerts its head-inhibiting action by way of the ventral nerve cords (Buchanan, 1922; Child and Watanabe, 1935; Watanabe, 1935*b*). Presumably the head-frequency gradient is the result of an interaction of these two opposed factors. Pieces from anterior levels may develop

[1] The writer wishes to express his appreciation to the Department of Zoölogy of the University of Chicago for the laboratory space and facilities which made this work possible.

[2] See Child (1941) for the most recent review and discussion of planarian reconstitution and experiments dealing with the control of correlative factors.

[3] This series of head forms in *Dugesia* has been repeatedly described and illustrated by various authors. See, e.g., Child, 1941, p. 178.

normal heads because of an initial high activity which permits them to escape any effects of posterior section, while pieces from posterior levels reconstitute into head-inhibited types because of a predominance of such inhibitory effects. It may be, however, that posterior inhibiting factors are less active at anterior levels. Toxic agents, such as KCN, appear to decrease the head frequency at anterior levels by direct action on the head-forming cells, while the head frequency of posterior pieces is increased by a greater inhibition or blocking of the posterior head-inhibiting factors in the very early stages of head regeneration.

MATERIAL AND METHODS

The material used in this investigation (*Dugesia dorotocephala*) was collected in large numbers from a spring near Cary, Illinois, during the summer of 1940. Large stocks were maintained under standard conditions (see Rulon, 1936) at the Zoölogical Laboratory of the University of Chicago. Preliminary experiments showed that whole unfed animals (14–16 mm. long) could survive for a few days in an M/8 solution of ethyl alcohol made up with well water, although it was often necessary to change the solution after the second day, when a few of the animals showed cytolysis in the head region. Since alcohol had been previously used in controlling head development in these forms (Child, 1911; Buchanan, 1922), it was selected as a test agent in the present experiments.

Reconstituted animals from pieces treated with alcohol were classified according to the following categories: (1) tailless, with normal or enlarged head; (2) near tailless, with normal or enlarged head; (3) bipolar, with head appearing at both the anterior and the posterior ends of the piece; (4) apolar, with neither a head nor a tail; (5) forms with normal tails but with heads ranging from normal through various medially inhibited types (teratophthalmic, teratomorphic, anophthalmic) to acephalic or headless (see Child and Watanabe, 1935); and (6) dead.

The approximate average degree of head inhibition in lots of pieces of a certain size and from a particular level was obtained by assigning an arbitrary value to each head type which roughly corresponded to the degree of inhibition (normal and bipolar, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; acephalic and apolar, 20).[4] The head-frequency index or mean of a control or experimental lot was calculated by multiplying the number of individuals of each head type by the number representing its degree of inhibition and dividing the sum of all by the total number of reconstituted forms from that level. When most of the worms developed normal heads, the index approached 100. When most were acephalic the index approached 20. This method for calculating the head-frequency index was not entirely satisfactory, since the assigned evaluation of head types according to degree of inhibition was only approximate. However, a large number of experiments and comparisons by different workers have shown it to be fairly reliable, particularly in comparing test and control experiments and in the comparison of reconstitution from different levels. Also, the head-frequency curves plotted from these indices are almost identical with those obtained by the use of more exact statistical methods (Castle, 1940).

[4] For convenience in recording, the different head types are given the following Roman numerals: normal, I; teratophthalmic, II; teratomorphic, III; anophthalmic, IV; and acephalic, V.

EXPERIMENTAL DATA

1. *The reconstitution of transverse pieces from animals treated with alcohol before section.*—Whole planarians, treated with M/8 alcohol for 3-5 days, became sluggish and responded slowly to external stimuli. When the anterior zooids of these animals were cut into transverse pieces, 1/8th the length of the entire animal (Fig. 1, *AB–IJ*), and the sections were allowed to reconstitute in well water, an increase in head frequency appeared at all posterior levels (Fig. 2, *L* = test, *M* = control). A few tailless, near tailless, and apolar forms appeared in the tests.

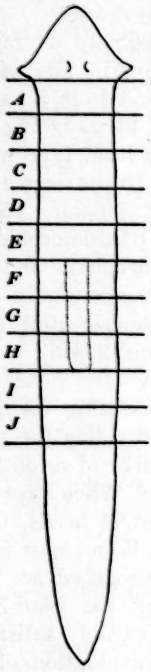

FIG. 1.—Sections approximately 1/16th the length of the intact animal are designated as *A*, *B*, *C*, etc., while sections 1/8th the length of the animal are designated as *AB*, *CD*, *EF*, etc., in all experiments.

When anterior and posterior regions of alcohol-treated animals were sectioned into pieces 1/16th the length of the entire animal (Fig. 1, *A*, *B*, *I*, *J*) and allowed to reconstitute in well water, not only was an increase in head frequency noted but a large number of forms with inhibited tails or bipolar heads appeared. This increased scale of organization is shown in Table 1, in which data on 50 pieces from *A* and *B* levels and 150 pieces (experiment performed three times with similar results) from *I* and *J* levels are given. The increase in head frequency was apparent at levels *A*, *I*, and *J*. Sections from *B* levels failed to show this increase because of a high percentage of apolar forms which were listed as acephalic in calculating the head-frequency index. The increase

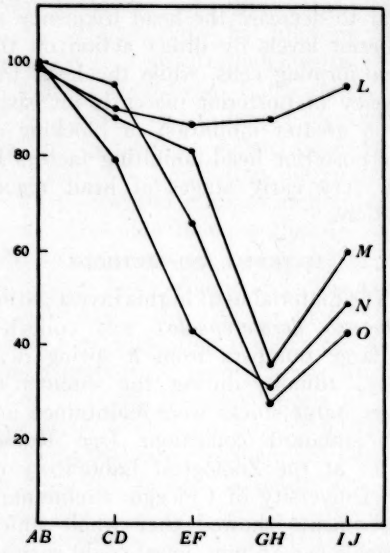

FIG. 2.—The effect of alcohol on head frequency of animals from pieces 1/8th the length of the intact planarian. Curve *L* represents treatment before section; *O*, after section; *N*, after, as well as before, section; and *M*, well-water control. The graph is plotted with the head-frequency indices as ordinates and with body levels as abscissae. Each curve represents at least 50 animals.

in forms with inhibited tails and normal or enlarged heads (except for the apolar forms, almost all of the animals with inhibited tails developed normal or enlarged heads) shows even better than the increased head frequency that this experimental treatment acts to increase the scale of organization (compare test and control, Table 1). Another interesting discovery was the increase in bipolar forms in levels *I* and *J* of the tests, which indicates an elimination or decrease in correlating factors within the pieces.

TABLE 1

THE EFFECTS OF M/8 ALCOHOL, ADMINISTERED 3–5 DAYS BEFORE SECTION, ON HEAD FREQUENCY AND SCALE OF ORGANIZATION IN ANTERIOR AND POSTERIOR 1/16TH PIECES

(Values in Percentage)

Section	Tailless	Near Tailless	Bipolar	Apolar	Head Types with Normal Tails					Dead	Index
					I	II	III	IV	V		
Control:											
A.....	24	10	0	6	36	4	4	0	0	16	91
B.....	6	4	0	10	10	0	4	14	20	32	50
I.....	1	5	2	7	9	2	3	7	55	9	39
J.....	1	2	3	6	12	2	5	7	62	0	39
Test:											
A.....	58	16	0	4	16	0	0	0	0	6	97
B.....	6	10	0	40	12	0	2	4	6	20	50
I.....	21	9	15	12	24	3	2	1	5	8	84
J.....	15	8	10	3	49	1	1	4	6	3	90

2. *The effect of alcohol on the development of pieces from untreated animals.*— Transverse pieces, 1/8th the length of the entire animal, were placed in M/8 alcohol for 72 hours directly after sectioning. They were then returned to well water for the remainder of the reconstitutional period. These experiments dealt with only the anterior five levels (Fig. 1, *AB*, *CD*, *EF*, *GH*, *IJ*). This treatment had little or no effect on *AB* pieces. Pieces from *CD* levels showed a slight increase, while *EF*, *GH*, and *IJ* showed a definite decrease in head frequency (Fig. 2, *O* = test, *M* = control). There was little difference between test and control as regards numbers of tailless, near tailless, apolar, or bipolar forms.

Transverse pieces, 1/16th the length of the intact animal, subjected to 72 hours' exposure to alcohol immediately after sectioning, gave interesting results. Test pieces from *B*, *C*, and *D* levels showed a definite increase in head frequency (Fig. 3, curve *W*) over the controls (Fig. 3, curve *X*). A 15 per cent increase in apolar forms caused a decrease in the head-frequency index of *A* levels. Test pieces from the six posterior (*E*, *F*, *G*, *H*, *I*, *J*) levels showed a definite decrease in head frequency (Fig. 3, curves *W* and *X*). The scale of organization in pieces from *A* and *B* levels was markedly increased in the tests, as shown in Figure 3 (curve *Y* = control, curve *Z* = test), where the percentage of forms with normal (uninhibited) tails is plotted against body level. Approximately 45 per cent of the controls, but only 10 per cent of the tests, had uninhibited tails. Although *C*, *D*, and *E* levels developed many with inhibited tails, there was little difference between test and control. Pieces from *F*, *G*, *H*, *I*, and *J* had few inhibited tails,

with little or no differences between test and control. There was no appreciable difference in numbers of bipolar or apolar forms with this treatment.

3. *The effect of alcohol on the development of pieces when administered before and after section.*—Whole planarians were treated with M/8 alcohol for 3–5 days. They were then sectioned into 1/8th pieces and left in the alcohol solution for 3 days before being transferred to well water. This treatment gave a decrease in head frequency in all but the most anterior level (Fig. 2, *N* = test, *M* = control). At *CD* level the tests also showed a slight increase in number of forms with inhibited tails.

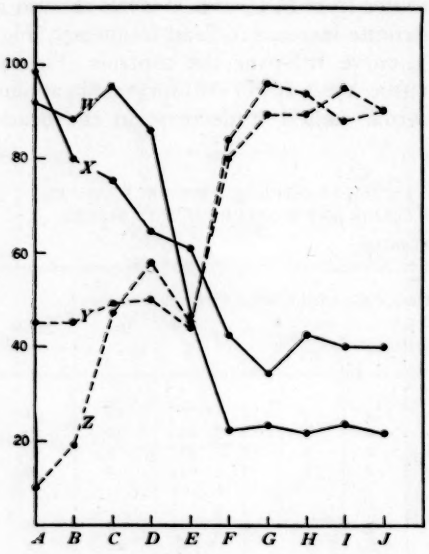

FIG. 3.—Curves *W* and *X* show the effect of alcohol when administered for 72 hours after sectioning on head frequency in 1/16th pieces (*W* = test, *X* = control). Curves *Y* and *Z* show the effect of alcohol when administered for 72 hours after sectioning on the reconstitution of normal tails in 1/16th pieces (*Z* = test, *Y* = control). For curves *W* and *X* head-frequency indices are plotted as ordinates and body levels as abscissae. For curves *Z* and *Y* percentages of normal tails are plotted as ordinates and body levels as abscissae.

DISCUSSION

Experiments by Rulon and Child (1937) in which short anterior pieces (1/16th the length of the whole animal) were subjected for 72 hours to M/100,000 KCN produced a decrease in scale of organization in the reconstituting animals. Levels which developed a high percentage of inhibited tails with normal or enlarged heads in well water gave more forms with normal tails and inhibited heads in the test solution. The development of tailless forms from short anterior pieces seems to be related to the high physiological activity of that level. Under such conditions the localization of more anterior parts takes place on a larger scale, leaving little or no tissue for the posterior or tail regions. Potassium cyanide appears not only to inhibit the reconstitution of these anterior pieces as a whole but to inhibit to a still greater extent the active anterior region of the reconstituting animal. This results in a decrease in dominance and a general flattening of the gradient within the piece. Under these conditions anterior parts are not favored, or may even be inhibited while posterior parts develop.

The development of partial (tailless) forms from short anterior pieces of planarians may be compared to that of partial or ectodermized echinoderm larvae produced from isolated animal halves of the early blastula (Hörstadius, 1935), which also show an increased scale of organization. Treatment of the animal halves of sea-urchin blastulae with lithium results in a decrease in scale of organization to such a degree that whole half-sized larvae may develop (von Ubisch, 1925, 1929). Such results

are not unlike those found when short anterior pieces of *Dugesia* are subjected to KCN, and indicate that similar physiological conditions are present in both embryonic and reconstitutional development.

Unfertilized echinoderm eggs, treated with NaCNS in Ca-free sea water for several hours, may develop into ectodermized forms when returned to normal sea water and fertilized (Lindahl, 1933, 1936; Rulon, 1941). These ectodermized forms are similar to those developing from animal halves alone and have been interpreted as being the result of an initial differential inhibition of the unfertilized egg which serves partially to obliterate the axiate pattern (Rulon, 1941). On return to sea water and fertilization the lower levels of the gradient, being wholly or partially physiologically isolated, may recover and develop in the same, or almost the same, manner as higher levels.

The reconstitution of tailless forms from short anterior pieces of *Dugesia*, taken from animals previously treated with alcohol (Table 1), indicates a loss of correlating factors in the pieces similar to that in echinoderm eggs treated with thiocyanate. Recovery and reconstitution in well water results in the more anterior parts being laid down on a larger scale at the expense of tail regeneration. The high percentage of tailless and bipolar forms developing in short pieces from the posterior levels (Table 1, *I* and *J*) results, on the one hand, from the effect of alcohol in decreasing correlative factors and, on the other, from more or less recovery from alcohol in the head-forming cells. This region of the body is at or near the fission zone where the gradient level changes from the low posterior end of the anterior zooid to the higher level at the anterior end of the second zooid (Child, 1941, pp. 108–11). In consequence of this change of gradient level, pieces from this region may have little or no gradient, or either end may represent a higher gradient level than the other. Since the fission zone is not directly visible, it is not possible to determine with certainty which pieces represent one or the other of these conditions; but the results of reconstitution give indicative evidence on this point and are readily interpreted on this basis. Bipolar forms develop from pieces in which any slight gradient differences present are so far obliterated by alcohol that, on return to water at the time of section, neither end is dominant over the other, both are essentially alike, and each develops independently of correlative factors and therefore gives rise to a head. In this connection Rustia's earlier work on bipolar forms is of interest (Rustia, 1925). In pieces with more gradient difference between the two ends, the gradient is not completely obliterated by alcohol; but it and the correlative factor, which originates at the posterior cut end of the piece and inhibits head development in varying degree, according to body level and length of piece, are decreased. Consequently, on return to water at the time of section, and upon more or less recovery from alcohol effects, the cells at the end of the piece representing the higher gradient level are less inhibited than in controls and determine the organization of the piece on a larger scale—often resulting in tailless forms.

With longer pieces (1/8th the length of the whole animal) the effect of alcohol, when administered before section, is most noticeable in a considerable increase in head frequency (Fig. 2, *L* = test, *M* = control). This also indicates a decrease or blocking of posterior head-

inhibiting factors, followed by recovery sufficient to permit all levels to reconstitute into normal animals. Treatment with M/8 alcohol for 72 hours after section, however, produced only a slight increase in head frequency at *CD* levels, while all posterior levels show a decrease (Fig. 2, *O* = test, *M* = control), although Buchanan (1922) showed that longer pieces in weaker solutions of alcohol gave an increased head frequency at posterior levels. Treatment before and after section with M/8 alcohol also gives a decrease in head frequency (Fig. 2, *N* = test, *M* = control). It appears that treatment after section with this concentration of alcohol is so severe that the head-forming cells do not recover, despite the fact that posterior head-inhibiting factors may be destroyed or blocked.

Short transverse pieces (1/16th the length of the whole animal) subjected to M/8 alcohol for 72 hours after section showed a slight decrease in head frequency at the most anterior level, an increase at the next anterior levels, and a marked decrease at the posterior levels (Fig. 3, *W* = test, *X* = control). It may be that the head-forming cells of anterior pieces from all but *A* levels are able to recover somewhat from the inhibitory effects of the treatment, while the head-forming cells of the posterior levels, because of an initial lower activity, are unable to do so. It is of interest to note that the more anterior levels give a higher percentage of tailless forms in the tests (Fig. 3, *Z* = test, *Y* = control), indicating recovery with increased scale of organization.

SUMMARY

1. Intact planarians (*D. dorotocephala*) were submitted to a well-water solution of M/8 ethyl alcohol for 3–5 days, sectioned into transverse pieces 1/8th and 1/16th the length of the entire animal, and the pieces allowed to reconstitute in well water. The reconstituted forms showed an increased scale of organization and head frequency in sections from all levels.

2. Exposure of freshly cut pieces from untreated animals for 72 hours after section to M/8 alcohol, followed by return to well water, resulted in an increase in scale of organization in anterior, and a decrease in head frequency in posterior, pieces.

3. Whole planarians, treated with M/8 alcohol for 3–5 days, sectioned into 1/8th pieces, and left in the alcohol solution for 3 days before transfer to well water, gave forms which showed a decrease in head frequency at all but the most anterior level.

4. The data indicate that the treatment of animals before section with alcohol results in a decrease or loss of correlating factors normally within the piece or arising at the time of section. This, together with a rapid recovery of head-forming regions on return to well water, may explain the increased scale of organization and head frequency.

5. The data also indicate that treatment of pieces during early reconstitution with M/8 alcohol inhibits—at least at posterior levels—the head-forming regions, despite the fact that other correlative factors in the piece may have been inhibited or blocked. The head-forming cells of anterior levels may recover if the inhibition is not too severe.

6. Attention is again called to the similarity of the factors underlying reconstitutional development in planarians and embryological development in echinoderms.

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THE RELATIONS BETWEEN NATURAL FISSION AND REGENERATION IN STYLARIA FOSSULARIS (ANNELIDA)

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A VERY extensive literature on the various aspects of annelid regeneration has been developed over the last forty years; but, unfortunately, much of this literature consists of reports of the axial difference in rate and potency of anterior regeneration. Very little importance has been attached to posterior regeneration. Natural fission, a phenomenon closely related to regeneration, has been adequately studied in naids in regard to the variations in the position of the fission zone and the frequency of fission at different levels along the body axis. However, the interrelation between the phenomena of fission and those of regeneration still remains obscure; nor is the nature of the distribution of natural fission zones and the significance of the frequency of fission at each level known. The present paper attempts to show that, at least in the case of *Stylaria fossularis*, a knowledge of the difference in the rate of anterior and posterior regeneration at different levels is of particular importance for an understanding of natural fission.

THE FREQUENCY OF NATURAL FISSION

According to Bourne (1891), the number of segments anterior to the fission zone is probably constant for each species. This suggestion was later denied by Stephenson (1930), owing to the fact that the number of these segments may vary from 12 to 20 in *Nais elinguis* and

from 13 to 29 in *Nais variabilis*. Piquet (1906) and Schuster (1915) considered this individual difference to be a seasonal variation. Later, Stolte (1923) reported that the asexual reproduction of naids was entirely a growth process, determined by nutrition, temperature, and the age of the animal. Eckert (1927, 1934), working with *Stylaria lacustris*, was able to confirm Stolte's opinion that nutrition is the most important factor in determining the position of the fission zone. Recently Van Cleave (1937) reached a similar conclusion from his experimental study of the fission process in *Pristina longiseta*, i.e., that the position of the fission zone can be altered by such enviromental factors as the state of nutrition, the temperature, and the age of the medium and that conditions favorable to the rapid growth of new segments produce intense fissioning activity, in the course of which the fission zones shift anteriorly.

The observations regarding the variations in the fission zone here reported were made on *S. fossularis* by the direct examination of worms collected from one pond. This study was carried out within 21 days (from March 17 to April 6), during which period the external environment probably remained fairly constant. For convenience of discussion, the term "frequency" will be used to denote the variation of level of fission zone. Table 1 summarizes the results thus obtained, in which the level was represented by the number of segments in the trunk region anterior to the fission zone and the frequency at each level was represented by the number of worms. In a total of 2,000 worms the variability in levels of fission ranged from segments 8 to 23, and the highest frequency hap-

TABLE 1

FREQUENCIES OF NATURAL FISSION AT DIFFERENT LEVELS IN 2,000 WORMS, WITH THE LEVEL REPRESENTING THE NUMBER OF SEGMENTS ANTERIOR TO THE FISSION ZONE

Level	Frequency											
	Date Counted										Total	In Percentage
	Mar. 17	Mar. 18	Mar. 20	Mar. 24	Mar. 25	Mar. 28	Apr. 1	Apr. 4	Apr. 5	Apr. 6		
8	.....	.....	.....	.....	.....	.....	.....	.....	.....	1	1	0.05
9	.....	1	.....	.....	.....	2	.....	.....	1	.....	4	0.20
10	2	.....	1	4	1	3	.....	2	3	.....	16	0.80
11	4	.....	4	4	2	4	3	4	3	1	29	1.45
12	7	6	6	5	2	9	7	3	11	6	62	2.60
13	13	6	16	14	6	10	10	10	17	12	114	5.70
14	14	9	15	19	17	8	19	16	18	24	159	7.95
15	28	6	18	22	23	26	16	17	34	25	215	10.75
16	29	9	27	28	23	26	23	23	43	31	262	13.10
17	28	23	28	22	22	28	28	31	58	34	302	15.10
18	25	14	20	35	17	32	34	50	61	49	337	16.85
19	19	7	14	24	24	36	25	38	64	50	301	15.05
20	13	1	14	14	11	15	14	10	28	27	147	7.35
21	2	5	2	6	4	5	3	3	8	3	41	2.05
22	1	1	.....	1	1	1	2	1	.....	1	9	0.45
23	.....	.....	.....	.....	.....	.....	.....	.....	1	.....	1	0.05
Total	185	88	165	198	153	205	184	208	350	264	2,000	100

pened to be at segment 18. These facts clearly demonstrate that the position of the fission zone is highly labile. To illustrate their distribution along the body axis, the data from Table 1 are plotted as a frequency curve (Fig. 1), where the abscissa represents the level and the ordinate represents the frequency. The curve shows clearly a double-gradient graph in which the frequency increases along the anteroposterior direction from level 8 to level 18. The latter represents the peak of the frequency curve. After this point the frequency decreases, so that fission rarely occurs in segments posterior to level 23. The theoretical consideration of these facts will be given at a later stage.

THE RATE OF ANTERIOR AND POSTERIOR REGENERATION

The process of regeneration occurring in *S. fossularis* is similar to that of *Dero limosa*, described by Hyman (1916). Worms with a certain number of segments (about 32–42 in the trunk region) and with no visible fission zone were selected. They were kept in the laboratory and starved for at least a week before use, in order that they might be in as nearly as possible the same physiological condition. A number of them were cut up at once; and, after cutting, the two parts of each worm were cultured in a single dish in boiled water, which was changed at regular intervals. Within a day or so, new tissues began to grow out from both cut surfaces. Anterior regeneration was being carried on from the cut surface of the posterior part of the worm; on completion of this process, there resulted a prostomium and other head segments (thoracic segments), whose bundles of setae appeared in an order along the anteroposterior axis. Furthermore, anterior regeneration of *S. fossularis* occurs in a very definite manner, which is characteristic of oligochaetes. If the number of segments removed was less than that of the head segments, they were replaced at once. If more were removed, then only these head segments were regenerated. In the meantime, posterior regeneration was being carried on from the cut surface of the anterior part of the worm. An anus appeared from an outgrowth in a few days; and, in front of it, new segments continued to be added indefinitely, much like the process of natural fission.

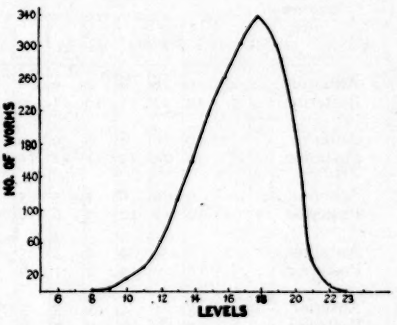

FIG. 1.—Graph showing the distribution of natural-fission frequencies among different levels.

The inability of the extreme anterior and posterior ends to regenerate has been noted by many investigators. In *S. fossularis* the anterior piece, consisting of only the head part (prostomium plus 4 thoracic segments), did not regenerate posteriorly unless it included 6 or 7 additional trunk segments. Those pieces consisting of less than this number almost invariably died within from a few hours to several days after operation, depending upon the number of segments present. When the level of cutting was on the 5th segment in the trunk, the posterior regeneration might proceed normally, producing a more or less limited number of new segments. But this number seemed to be usually less than is

sufficient for its continued existence, and the worm eventually died. When the cutting was at level 6, complete posterior regeneration generally resulted, giving new worms in most cases. Sometimes the regenerates, however, suffered the same fate as did the worms which were sectioned at level 5. When the cutting level was beyond level 6, the posterior regeneration proceeded without any occurrence of anterior regeneration. Since anterior regeneration did not occur at level 26, this minimum number may be roughly estimated at 10–15 segments.

From the above description it is obvious that both anterior and posterior complete regeneration can be brought about between levels 6 and 25. The authors regret, however, that, owing to the lack of temperature-control equip-

TABLE 2

RATES IN DAYS OF ANTERIOR AND POSTERIOR REGENERATION

Regeneration	Level																			
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Anterior	4	9	8	8	7	9	8	9	8	9	8	8	8	10	10	8	11	10	10	7
Posterior	7	14	12	11	10	11	9	10	9	10	9	9	9	10	9	7	8	8	7	5
Anterior	...	9	8	8	8	9	9	7	9	9	9	9	8	10	11	10	10	9	...	10
Posterior	...	15	13	12	11	12	11	8	10	10	9	9	8	9	9	8	8	7	...	6
Anterior	...	9	9	8	4	5	5	8	7	7	9	9	9	6	7	10	9	7	...	...
Posterior	...	16	12	12	5	6	6	9	8	7	10	9	8	6	6	8	7	5	...	...
Anterior	...	5	4	4	4	5	5	6	8	7	8	10	6	6	6	6	...	10	...	...
Posterior	...	8	6	5	6	7	6	7	9	8	9	9	6	5	5	5	...	7	...	...
Anterior	...	5	4	4	5	5	5	6	9	...	9	8	6	6	...	9	...	7	...	...
Posterior	...	7	6	6	7	7	6	7	10	...	9	9	6	5	...	7	...	5	...	...
Anterior	...	4	4	4	...	...	...	...	...	...	8	8	6	10	...	...	...	...	...	...
Posterior	...	7	6	6	...	...	...	...	...	...	8	8	7	10	...	...	...	...	...	...
Anterior	...	...	...	...	...	...	...	...	...	...	...	...	8	...	...	...	...	...	...	...
Posterior	...	...	...	...	...	...	...	...	...	...	...	...	8	...	...	...	...	...	...	...
Anterior	...	...	...	...	...	...	...	...	...	...	...	...	7	...	...	...	...	...	...	...
Posterior	...	...	...	...	...	...	...	...	...	...	...	...	6	...	...	...	...	...	...	...

difficulty, varying in rate at different levels, as will be illustrated in later sections.

For anterior regeneration the situation was somewhat different. Although the short posterior pieces consisting of less than a certain number of segments would not regenerate anteriorly, they remained alive for about a month. As a result of the unlimited growth of the posterior part of the animal, it seems impossible to determine the minimum number of segments necessary for the ment, unavailable in a wartime laboratory, which is deficient in many ways, the respective rates of regeneration cannot be directly measured in time units. Their difference was, therefore, expressed in terms of a relative rate, which may be designated as the ratio between the rates of anterior and those of posterior regeneration. We have already seen that the two parts of a worm, after cutting, were cultured in one dish under the same external conditions—a procedure that made their subsequent rates

of regeneration comparable. For the sake of simplicity, the relative rate of anterior regeneration was expressed by the ratio Ra/Rp and that of the posterior regeneration by the ratio Rp/Ra, with Ra and Rp being the rates of anterior and posterior regeneration, respectively.

Table 2 gives the observed rates of both the anterior and the posterior regeneration in days, with the time required for the completion of regeneration in reference to an arbitrarily chosen standard. In all cases, only the living pieces were considered, and experiments containing a large number of deaths were discarded. The relative rates calculated from the data available are pre-

TABLE 3

RELATIVE RATES OF REGENERATION CALCULATED FROM THE DATA SHOWN IN TABLE 2*

$$\left(\text{Anterior} = \frac{Ra}{Rp}; \quad \text{posterior} = \frac{Rp}{Ra}\right)$$

Regeneration	Level																			
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Anterior	0.57	0.64	0.67	0.73	0.70	0.82	0.90	0.90	0.90	0.90	0.90	0.90	0.90	1.00	1.11	1.14	1.38	1.25	1.43	1.40
Posterior	1.75	1.56	1.50	1.38	1.43	1.22	1.13	1.11	1.13	1.11	1.13	1.13	1.13	1.00	0.90	0.88	0.73	0.80	0.70	0.71
Anterior	.....	0.60	0.62	0.67	0.73	0.75	0.82	0.88	0.90	0.90	1.00	1.00	1.00	1.11	1.22	1.25	1.25	1.29	.....	1.67
Posterior	.....	1.67	1.63	1.50	1.38	1.33	1.22	1.14	1.11	1.11	1.00	1.00	1.00	0.90	0.82	0.80	0.80	0.78	.....	0.60
Anterior	.....	0.56	0.75	0.67	0.80	0.83	0.83	0.90	0.88	1.00	0.90	1.00	1.13	1.00	1.17	1.25	1.20	1.40	.....	.....
Posterior	.....	1.78	1.33	1.50	1.25	1.20	1.20	1.13	1.14	1.00	1.11	1.00	0.90	1.00	0.86	0.80	0.78	0.71	.....	.....
Anterior	.....	0.63	0.67	0.80	0.67	0.71	0.83	0.86	0.90	0.88	0.90	1.11	1.00	1.20	1.20	1.20	.....	1.43	.....	.....
Posterior	.....	1.60	1.50	1.25	1.50	1.40	1.20	1.17	1.13	1.14	1.13	0.90	1.00	0.83	0.83	0.83	.....	0.70	.....	.....
Anterior	.....	0.71	0.67	0.67	0.71	0.71	0.83	0.86	0.90	.....	1.00	0.90	1.00	1.20	.....	1.29	.....	1.40	.....	.....
Posterior	.....	1.40	1.50	1.50	1.40	1.40	1.20	1.17	1.11	.....	1.00	1.13	1.00	0.83	.....	0.78	.....	0.71	.....	.....
Anterior	.....	0.57	0.67	0.67	.....	.....	.....	.....	.....	.....	1.00	1.00	0.86	1.00	.....	.....	.....	.....	.....	.....
Posterior	.....	1.75	1.50	1.50	.....	.....	.....	.....	.....	.....	1.00	1.00	1.17	1.00	.....	.....	.....	.....	.....	.....
Anterior	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1.00	.....	.....	.....	.....	.....	.....	.....
Posterior	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1.00	.....	.....	.....	.....	.....	.....	.....
Anterior	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1.17	.....	.....	.....	.....	.....	.....	.....
Posterior	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	0.86	.....	.....	.....	.....	.....	.....	.....
Average:																				
Anterior	0.57	0.62	0.68	0.70	0.72	0.76	0.84	0.88	0.90	0.92	0.95	0.99	1.01	1.09	1.18	1.23	1.31	1.35	1.43	1.54
Posterior	1.75	1.63	1.49	1.43	1.39	1.31	1.19	1.14	1.12	1.09	1.06	1.03	1.01	0.93	0.85	0.80	0.77	0.74	0.70	0.66
$\frac{RRa+RRp}{2}$	1.16	1.13	1.09	1.07	1.06	1.04	1.02	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.02	1.02	1.04	1.05	1.07	1.10

* Explanations in text.

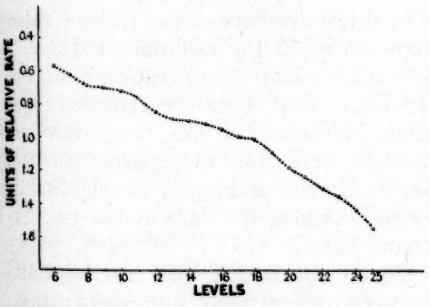

Fig. 2.—Gradient curve of anterior regeneration

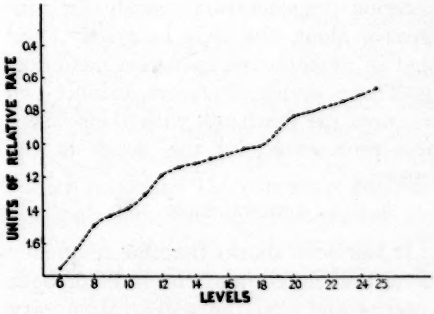

FIG. 3.—Gradient curve of posterior regeneration

sented in Table 3. This table shows that the value of the relative rate of anterior regeneration increases as the cutting level rises and that the reverse is true for posterior regeneration. An increase in the value of the relative rate means a decrease in the actual rate of regeneration. For example, in the first line of Table 3 we have a value of 0.67 at level 8 and 1.38 at level 22 for anterior regeneration. If two worms were now sectioned at levels 8 and 22, respectively, the posterior part in the former

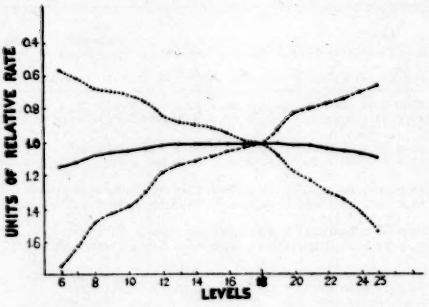

FIG. 4.—Combination of Figs. 2 and 3, showing the location of equal relative rates for both anterior and posterior regeneration at level 18. The meaning of the horizontal line plotted from the mean average rates is discussed in the text.

case would regenerate more quickly than that in the latter. Furthermore, this important character can be seen clearly by plotting the average values in Table 3 into graphs (Figs. 2 and 3). The rate of anterior regeneration gradually increases along the axis anteriorly, and that of posterior regeneration posteriorly. These worms, therefore, exhibit two regeneration gradients with their steepness represented by the slopes of the curves.

DISCUSSION

It has been shown that the frequency of natural fission and the rates of both anterior and posterior regeneration vary with different levels. By combining Figures 2 and 3, we obtain Figure 4, in which the intersecting point of the two curves represents a level possessing the same relative rate for both types of regeneration. It is interesting to note that this intersecting point falls at level 18, which is the highest point of the frequency curve of natural fission.

The gradient curves of regeneration indicate not only the differences in the rate of regeneration but also, in general, the differences in potency of regeneration among different levels. Consequently, these curves also roughly represent the curves of potency of regeneration. Level 18 is thus the level at which the potency ratio between the anterior and posterior regeneration approaches unity. At any other level it may become either more or less than unity, but the sum of the two regeneration potencies remains the same. In terms of relative rate, this relation may be expressed by the formula $RRa + RR\rho = K$, where RRa represents the relative rate of anterior regeneration, $RR\rho$ the relative rate of posterior regeneration, and K is a constant. If our reasoning is correct, at any level both the sum of the two regeneration potencies and the sum of the relative rates, when divided by 2, should be equal to K. The conclusion could be reached from the mere inspection of the curves, but it can also be more profitably approached by the following deduction: The mean averages of the relative rates represented in the last line of Table 3 give a horizontal line, as shown in Figure 4; this is what would be expected. The slight deviation of this line from the straight line was due partly to the sampling error and partly to the difficulty of obtaining the data at the two extreme ends.

Before discussing the interrelations between natural fission and regeneration, a few words seem necessary to explain

how a calculated frequency curve can be obtained. Table 4 shows the regeneration indexes which were calculated on the basis of *RRa/RRp* for levels before level 18 and of *RRp/RRa* for those behind it. When these values are plotted against the levels, we obtain an index curve (Fig. 5) which is similar in general trend to that of Figure 1. Now, by taking the highest frequency of natural fission of the observed 2,000 worms (i.e., 337 at level 18) as a standard, the calculated frequency at any other level can easily be obtained from the regeneration index at the level in question; this is also shown in Table 4. From these values we obtain another curve, the calculated frequency curve, which is plotted by a dotted line in Figure 6, together with the observed-frequency curve. The increase in frequency at levels other than levels 18 and 19 in the calculated curve is apparently a result of the wound stimulation of cutting. The similarity of these two curves is, however, noteworthy. It clearly points out a possibility that certain common factors may be involved in the processes of both fission and regeneration; but what these factors are, is still an open question.

The process of fission has generally been regarded as the result of physiological isolation (Child, 1924). As the body size of a worm exceeds a certain limit, physiological isolation occurs. The fate of the physiologically isolated parts depends upon their own potencies. They may be either capable of regeneration or incapable, with atrophy and ultimate death resulting. The process of regeneration after cutting may be viewed as a physical isolation; and the fate of the physically isolated parts, therefore, also depends on their own potencies.

As regards the determination of the

TABLE 4

REGENERATION INDEXES AND CALCULATED FREQUENCIES OF FISSION REPRESENTED BY NUMBER OF WORMS*

	Level																			
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Index	0.33	0.38	0.43	0.49	0.52	0.58	0.71	0.77	0.80	0.84	0.90	0.96	1.00	0.85	0.72	0.65	0.59	0.55	0.49	0.43
No. of worms	110	128	145	165	175	196	238	260	271	284	302	324	337	387	243	219	195	185	165	144

* The number 337 at level 18 is taken as standard.

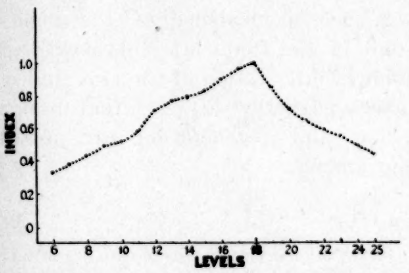

FIG. 5.—Graph showing the distribution of the calculated fission frequencies obtained from regeneration indexes.

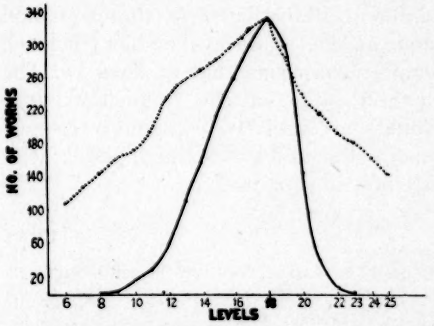

FIG. 6.—Graph showing the general similarity between the observed and calculated frequency curves.

position of the fission zone, Hempelmann (1923) believed that in *P. longiseta* some undefined internal factors played a significant role. From the data presented in this paper it is evident that natural fission could be interpreted in either of the two following alternatives: (1) Natural fission may probably occur at any level between the 6th and the 26th trunk segment. The distribution of the position of the fission zone is merely a problem of chance. The fact that fission occurs most probably at level 18 is possibly related to the peculiar property of that level, i.e., its equal distribution of anterior and posterior regeneration potencies. (2) Natural fission can take place only at a definite level. This level, which may also be the region of equal distribution of two regeneration potencies, may vary among different individuals, so that a fission zone at level 15 or level 22 has much the same meaning as that at level 18. The highest point of the frequency curve could be similarly explained. Experiments designed to test these possibilities are now in progress.

SUMMARY

1. The variation in the position of the natural fission zone in *S. fossularis* may vary from level 8 to level 23, and the maximum frequency obtained from an analysis of a total of 2,000 worms occurs at level 18.

2. Within certain limits, the rate of anterior regeneration increases gradually toward the anterior end of the worm, and that of posterior regeneration toward its posterior end.

3. The two regeneration gradients meet at level 18, which may be regarded as a balance-point in the sense of equipotency for both regenerations. Accordingly, cutting at this point will invariably lead to the result that anterior and posterior regeneration will proceed at an equal rate.

4. The coincidence of the highest point of the frequency curve with the point of intersection of the two gradient curves naturally suggests that natural fission and regeneration are related phenomena.

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THE MIGRATION OF MELANOBLASTS AFTER HATCHING INTO PIGMENT-FREE SKIN GRAFTS OF THE COMMON FOWL[1]

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BY TRANSPLANTING melanoblasts of birds from one embryo to another of a genetically different breed (Rawles, 1939; Willier and Rawles, 1940) it has been possible to obtain information which throws considerable light on the behavior of melanophores and their role in the production of color and pattern in feathers. Results from an extensive series of such transplantations, in which embryos of many common breeds of fowl and other birds, including several of the song birds, were used, have shown quite clearly that donor melanoblasts migrate from their site of implantation into the surrounding host tissues and enter the developing feathers of the host. Invariably they produce in the host feathers, regardless of breed, not only their specific color but also their specific pattern—i.e., barring, spotting, penciling, etc.

As a rule, this migration during embryonic development is very extensive. Frequently an implant of head or somite skin-ectoderm, or pure mesoderm (Watterson, 1938), of less than a square millimeter inserted in an incision at the base of the prospective wing region (at approximately 70 hours' incubation), furnished sufficient melanophores to produce donor color in the feathers of the entire host wing and even adjacent tracts. While the donor-colored area was, on the whole, less widespread in the juvenile plumage than in the down, the effect, nevertheless, did persist in the juvenile feathers, sometimes throughout their entire development. In many cases, however, host pigment began to show up in the bases of the juvenile feathers before their development was completed, particularly in those emerging later, producing mosaics of various types. These were always followed by feathers showing host pigment entirely. Therefore, it is apparent that, after hatching, the foreign (donor) melanophores are gradually replaced by those belonging to the host.

Danforth (1929*a*) and Danforth and Foster (1929) noted only a very slight migration of host melanoblasts when skin grafts were made between different breeds of fowl at hatching or shortly afterward. The mosaic feathers which were obtained were not numerous and were confined to the graft margin. The vast majority of the grafted feathers maintained their original (donor) color and pattern throughout the life of the bird. Why the migration of host pigment cells should be so restricted in these cases and so unrestricted in ours brings up an interesting problem. Do the melanophores which are already present in

[1] Preliminary experiments for this report were carried out at Stanford University in 1940 during a visit of four months in Professor C. H. Danforth's laboratory. The writer takes great pleasure in acknowledging her indebtedness to Professor Danforth for placing the facilities of his laboratory at her disposal, for personal instruction in the skin-transplantation technique, for friendly encouragement, and for the interest with which he has followed the experimentation since its beginning. Throughout the course of the work Professor B. H. Willier has been a constant source of help. It is with pleasure that the author acknowledges her indebtedness to him also. For the photographs of Plates I and II the author is indebted to Mr. John Spurbeck.

a piece of skin grafted late—i.e., at hatching or a few days afterward—furnish a block to those of the surrounding host tissues? Would host melanophores enter the developing feathers of a skin graft deprived of its normal complement of melanophores? The experiments reported in this paper were designed to test this question.

In order to obtain fully differentiated pigment-free (white) down plumage from pigmented breeds of fowl, it is only necessary to remove a desired portion of the embryo—such as a limb bud, for example—before the neural-crest cells migrate into it, and to place it in a site where it will subsequently grow and differentiate normally but will not take up pigment-forming cells. The experiments of Eastlick (1939) and Ris (1941) have shown that the embryonic coelom of the White Leghorn fulfils these requirements quite satisfactorily. It seemed possible that a retransplantation of such experimentally produced white down feathers to newly hatched chicks of various pigmented breeds as second hosts might afford a means for acquiring information of a more definite kind regarding the migration of melanophores after hatching. The method has proved most useful, not only for demonstrating a continuous and extensive posthatching migration of melanophores, but also for the opportunity it furnishes for analyzing the separate roles of the melanophore and the feather germ in pattern formation.

THE GENERAL PROCEDURE

In the present experiments the wing bud was chosen for isolation for several reasons. First, the time when the neural-crest cells migrate into it has been established by our own experiments and those of Watterson (1942); second, the wing, more than any other portion of the body, affords the widest variety of feather types; and, last, one wing bud can be removed easily without disturbing the normal development of the embryo—a circumstance to be taken into account when one wishes to carry the donor through hatching—while the wing bud on the opposite side serves as a control.

In general, the procedure was as follows: the right wing bud was removed, totally or in part, from donor embryos of pigmented breeds (New Hampshire Red and Barred Plymouth Rocks chiefly) of approximately 65–78 hours' incubation (28–38 somites), i.e., before the neural-crest cells had migrated into it (Pl. I, Fig. 1), and transferred through an incision in the somatopleure to the coelom of a White Leghorn host embryo of 60–70 hours' incubation age (Pl. I, Fig. 2). In this favorable site the transplant grows and differentiates into a wing or a part of a wing, depending upon whether the whole bud or only a portion was isolated originally, and is covered with structurally normal nonpigmented (white) feathers (Pl. I, Fig. 4). At hatching or soon afterward, the graft was removed from the wall of the body cavity of the White Leghorn host, where it had become attached; and a piece of skin, as nearly rectangular as possible, was dissected away from the underlying muscle and bone. This piece of skin with its white plumage (down plus the primordia of the juvenile flight feathers) was now retransplanted to the back region of a newly hatched chick (host No. 2) of any desired breed, usually pigmented, from which an area of back skin approximately the size of the graft had been removed (Pl. I, Fig. 5). The graft was sewed into place with silk thread. Care was taken to fit the cut margins of the host and graft skin neatly together without rolling or overlapping the edges.

As a rule, Barred Plymouth Rock and New Hampshire Red chicks were used as hosts for the second transplantation, but in a few cases transplants were made to White Leghorn and F_1 hybrid chicks from a Barred Plymouth Rock–New Hampshire Red cross. In one series the original donors, after hatching, served as the second hosts.

Since the two transplantation techniques employed in this experimentation are not new and have been described before, nothing further will be said about them here. For technical details concerning the first, intracoelomic grafting, the reader is referred to Hamburger (1938, 1939), who originated the technique, to Eastlick (1939), and to Rawles (1940); the second, concerned with transplanting skin of newly hatched chicks, is described fully by Danforth and Foster (1929).

THE INTRACOELOMIC GRAFTS

The growth and differentiation of limb buds in the embryonic coelom of the chick have been described in detail by both Hamburger (1938, 1939) and Eastlick (1939). Since the present data are, on the whole, in good agreement with theirs and bring out no particularly new features, no description of the grafts will be attempted. In the present work the method of grafting to the coelomic cavity was employed solely for the purpose of procuring an area of skin with fully differentiated, pigment-free down plumage which could be retransplanted at hatching to the back of a newly hatched host chick, and in this respect it has served its purpose admirably.

The White Leghorn was chosen for the host embryo because the epithelial lining of its coelomic wall is practically melanophore-free, in contrast to that of other common white breeds, such as the White Plymouth Rock and White Wyandotte. Occasionally, however, pigment is found in the White Leghorn coelom. A careful examination of the coelomic walls of several hundred White Leghorns near the end of the incubation period (18–20 days) has shown that, in approximately 5 per cent of the cases examined, traces of pigment can be found either in the form of a few expanded melanophores containing small black granules or, more often, as small blobs of black granules which appear to be degenerated melanophores. When pigment is present, it is nearly always more abundant in the region of the umbilicus (Ris, 1941). It may be that the pigment disappears altogether from the coelom shortly after hatching. Examination of the coelomic lining has not been made during this period. While pigment is found rather infrequently in the coelomic epithelium near hatching, the down feathers of the body, on the other hand, at the same period always contain a generous supply of melanophores, filled with small black granules, confined at the base of the feather, forming a characteristic ringlike configuration (Willier and Rawles, 1940). In some individuals these pigment rings are very dark and conspicuous; in others, quite faint. In all cases they can be seen if looked for carefully.

Concerning the wing grafts, it is known from the experiments of Eastlick (1939) and of Ris (1941) that a graft growing in the coelom will take up pigment-forming cells from a pigmented host if it becomes attached to the inner body wall. In the present experiments most of the grafts were firmly attached to the dorsal or dorsolateral body wall at the leg level, rather than to the mesenteries, and thus were favorably located for receiving pigment cells from the White Leghorn host if any should be present. Of the 115 pieces of skin bearing white down that were dissected from the

wing grafts for retransplantation, 50 showed traces of White Leghorn melanophores in their typical ringlike formation at the bases of a few of the white feathers. In no case had the White Leghorn melanoblasts penetrated all the developing feathers of any one graft. The pigment rings were intense enough to be conspicuous in only 3 or 4 cases. In the great majority they could have been overlooked easily if they had not been especially looked for. The skin between the follicles was usually free of any pigment formation. The presence of White Leghorn melanophores in no way affected the results after retransplantation to the second host. Pigment cells from the back region of the host (No. 2) migrated into the feathers of the transplant and produced in them a typical host pattern.

At the time of the operation the donors had well-defined wing buds. The majority would fall into Stage 5 of Hamburger's (1939) classification; i.e., length :width = 4:1. A few, slightly older, would come nearer Stage 6: length: width = 2.5:1. Although the further differentiation of wing buds isolated at such late stages is more likely to be atypical than when the isolation is made earlier (cf. Hamburger, 1939), the older buds can, nevertheless, be more easily removed and with less injury to the donor.

The size of the piece of skin recovered for transplantation after differentiation in the White Leghorn coelom depended upon whether the whole limb bud or only a part was removed originally. When the bud is divided, the skin ectoderm has a tendency to separate from the mesenchyme and to roll up into a tube and differentiate into a hollow vesicle with the feathers growing toward the interior of the sac. In such cases the vesicle of differentiated skin and feathers can be slit, opened, and spread for sewing on the back of the young host chick. But the piece of skin is usually small—on the average, ½ cm. in diameter—and rather tedious to handle. Therefore, this procedure of isolating portions of the bud was abandoned, and the whole bud was taken in the later experiments. When the entire wing bud was grafted, a piece of skin 0.5–1.0 cm. × 1.5–3.0 cm. can, as a rule, be recovered for retransplantation. Some of the area of skin was necessarily lost in straightening up the edges preparatory to fitting the transplant in place properly on the host back. Owing to the narrowness of the hand or tip region and the large size and rigidity of the developing primaries, both of which increased the difficulty of manipulation, the tip of the wing was often discarded. Therefore, in the majority of cases the transplant was reduced to include the skin from the ulnoradial region plus the adjacent humeral portion.

SERIES 1. THE RETRANSPLANTATION OF WING SKIN DIFFERENTIATED IN THE WHITE LEGHORN COELOM TO THE ORIGINAL DONOR CHICK

In this series of experiments, consisting of 15 cases (14 Barred Plymouth Rocks and 1 New Hampshire Red), the pieces of wing skin dissected from the graft after its 17 or 18 days' growth in the White Leghorn coelom were retransplanted to the back region of the original donors soon after hatching, when their down had become dry and fluffy. The donor chicks hatched with the right wing missing entirely or in part, depending upon whether the whole bud or only a portion of it had been removed originally. Those that hatched were quite as normal and vigorous as unoperated chicks of the same age.

Since the wing bud had been removed from the donor embryo before the neural-crest cells had migrated into it, and thus

was deprived of its normal source of melanoblasts, no melanophores ever appeared in the feathers which developed on the wing graft during its residence in the White Leghorn coelom. Consequently, the down feathers and the portions of the juvenile flight feathers which had formed prior to hatching were always unpigmented, despite the fact that they had come from donors of a pigmented breed.

TABLE 1*

A SUMMARY OF THE DATA ON GRAFTING PIGMENT-FREE SKIN

Series	Breed of Donor (Embryo—65–78 Hours' Incubation)	Breed of Host I (Embryo—60–70 Hours' Incubation)	Breed of Host II (Newly Hatched Chick)	No. of Chicks Which Died Early (within 2 Weeks after Retransplantation)	No. of Grafts Which Failed		No. of Grafts Which Persisted	Total No. of Cases
					Before Producing Feathers	After Producing Feathers		
I.....	B.P. Rock	W.L.	B.P. Rock (original donor)	2	1	0	11	14
I.....	N.H. Red	W.L.	N.H. Red (original donor)	0	0	0	1	1
II.....	B.P. Rock	W.L.	N.H. Red	2	29	5	1	37
II.....	B.P. Rock	W.L.	B.P. Rock	4	23	13	4	44
II.....	B.P. Rock	W.L.	W.L.	0	0	2	0	2
II.....	N.H. Red	W.L.	B.P. Rock	0	8	4	1	13
II.....	N.H. Red	W.L.	F_1 Hybrid	0	0	0	1	1
II.....	W.L.	W.L.	B.P. Rock	0	2	1	0	3

* B.P. Rock = Barred Plymouth Rock; N.H. Red = New Hampshire Red; W.L. = White Leghorn.

SERIES 2. THE RETRANSPLANTATION OF WING SKIN DIFFERENTIATED IN THE WHITE LEGHORN COELOM TO OTHER NEWLY HATCHED CHICKS OF THE SAME OR OF A DIFFERENT BREED

The important difference between these experiments and the preceding is that the piece of skin dissected from the intracoelomic wing graft was retransplanted to the back region of normal host chicks of the same or of a different breed instead of being returned to the original donor. In the majority of cases this second host belonged to a different breed. Of the 100 cases which make up this series, 37 represent wing skin from Barred Plymouth Rock donors transplanted to New Hampshire Red hosts; 13 from New Hampshire Red donors to Barred Plymouth Rock hosts; 1 from a New Hampshire Red to an F_1 hybrid host (from a cross between a Barred Rock female and a New Hampshire Red male); 2 from Barred Plymouth Rock to White Leghorn hosts; and 3 from White Leghorn to Barred Plymouth Rocks. In the remaining 44 cases the transplants came from Barred Plymouth Rock embryos and were grafted to other individuals of the same breed.

In this series, as in the former, the donors were usually saved and were allowed to continue their development as long as possible. While the majority did not hatch, many developed far enough to permit their sex to be determined.

RESULTS

A summary of the data is given in Table 1, where it is seen that 115 individuals received transplants of wing skin bearing white feathers which had undergone growth and differentiation in

the White Leghorn coelom. Series I includes the 15 cases (14 Barred Plymouth Rock; 1 New Hampshire Red) in which the transplants were returned to the original donor after it had hatched. Series II includes the cases (100 in number) in which the pieces of wing skin were retransplanted to normal individuals of the same breed (44 cases) or of a different breed (56 cases). As regards the characteristics of the feathers produced by the transplants, the results of the two series are in perfect agreement; but, in regard to the reaction between the tissues of the donor and the host, the results of the two series differ greatly. When the donor and host represent different individuals of the same breed or of another breed (as in Ser. II), we are confronted with the problem of tissue antagonism or incompatibility, which has always presented certain difficulties whenever heterografts among animals in general have been attempted.

HOST-GRAFT RELATION

No tissue antagonism of any kind was observed in the autoplastic transplantations of Series I. Whenever the piece of skin is returned to the same genotype (original donor), one can be sure of getting a "take." The one failure which occurred in this series was undoubtedly the result of faulty technique. Of the two chicks that died early—one at 3 days, the other at 12 days—the latter presumably would have produced feathers, since the transplant had healed perfectly and the transplanted skin appeared normal in every respect. We see, therefore, that 12 of the 15 transplants returned to the back region of the original donors persisted and continued to produce wing feathers so long as the birds remained under observation. In 4 cases this covered a period of nearly 3 years. Only 2 individuals died under 1 year of age, and so the majority lived long enough to pass through at least one natural molt of adult plumage.

When these results are compared with those from Series II, where the retransplantation was made to hosts other than the original donor, we find striking differences in the number of "takes" obtained. One hundred individuals are included in this series (see Table 1). Six died early (i.e., within the first 2 weeks), without giving any precise information as to the ultimate fate of the transplant, and therefore may be disregarded. This leaves 94 individuals with pertinent records. If we consider the production of feathers by the graft as the criterion of success, then 32 cases, or 34 per cent, of the transplants were successful. But 25 of the 32 grafts which produced feathers ultimately regressed during a period of from 1 to 4 months after transplantation. In one exceptional case the transplant persisted for $7\frac{1}{2}$ months and produced several generations of feathers (induced by plucking) before it was finally shed. However, this case had shown evidence of tissue incompatibility 3 months previously, but recovery set in and normal feathers were produced up until the final "attack" at $7\frac{1}{2}$ months. Fortunately, each breed-combination was represented among the 7 cases in which the grafts persisted. The transplants of 3 of these 7 individuals—namely, New Hampshire Red skin to Barred Plymouth Rock and the reciprocal, and New Hampshire Red skin to F_1 hybrid—are now being followed into their third year. The four Barred Plymouth Rocks which received transplants from other Barred Plymouth Rocks were followed continuously for 1–2 years, when they either died or had to be killed because of illness. The grafts on these individuals never exhibited any signs of tissue antagonism but continued to produce feathers in a perfectly normal manner; so there seems no doubt as to their permanence.

The data show that 66 per cent of the grafts failed before producing wing feathers (Table 1, sixth column). The time of failure varies considerably, ranging from approximately 1 to 3 weeks. While no entirely adequate explanation of the large number of failures can be offered, it is possible that factors other than tissue incompatibility—such as size of piece, imperfections in technique, etc.—may play a part. In some of the earlier experiments, where the wing bud was divided before implantation to the coelom, the pieces of skin recovered were very small (not more than a centimeter in length, sometimes less) and consequently were difficult to sew on properly. At best, the skin transplants were never large. From a well-developed intracoelomic wing graft the piece of skin obtained, after dissection and trimming, would average scarcely more than 2 cm. in length by 1 cm. or less in width. The fact, further, that the skin which differentiates in the coleom until hatching appears thinner and more tender than that which develops on the outside of the body, together with the size and rigidity of the developing flight feathers, increased the difficulty of fitting and suturing the transplant in place without splitting and tearing.

In general, the results indicate that the larger transplants have better chances of survival. Nearly all grafts "take" in the sense that they heal in, and become a part of, the host for a period of at least a week. During this time they look alike, and it is impossible to predict their fate. The failures take several different forms. Among those grafts shed early—within the first 3 or 4 weeks before producing pin feathers—the first signs of failure were often an edematous reaction in some portion of the skin, accompanied by the exudation of a yellowish fluid and followed by necrosis and scab formation, which increased until the whole surface was affected and eliminated (cf. Eastlick, 1941). In others, no particular skin reaction was observed; but the whole area became dry and hard and was shed as a whole or in bits. Among grafts of longer standing which had begun to produce contour feathers, failures likewise followed no prescribed pattern. In some cases the first signs of regression appeared in the feather germs. The feathers actually wilted at their bases and fell from the follicles, while the surrounding skin appeared to be perfectly normal. Sometimes after a few weeks of latency the feather germs recovered and resumed their normal function, but such recovery was nearly always temporary. Sooner or later, the whole area was lost. In other cases the first signs of regression appeared in the skin as a whole, with an edematous reaction, followed by necrosis and scab formation and ultimate elimination of the graft area. At any stage, regression may be slow and gradual or rather sudden. It is possible that these differences in the form and time of the reaction may represent degrees of incompatibility between donor and host tissues.

The ages at which failures become apparent have shown such a wide variation that it is very difficult to make a summarizing statement regarding the critical periods. However, in general, transplants that reach the age of 3–4 weeks without having shown signs of incompatibility have a fair chance of producing feathers, while those surviving for from 3 to 4 months stand a very good chance of remaining permanently.

THE CHARACTERISTICS OF THE FEATHERS PRODUCED BY THE SKIN GRAFT

In all of the 44 cases that produced feathers (see Table 1), the white down plumage was replaced by wing contour feathers typical of the portion of the wing from which the transplant was

taken. Usually this was the ulnoradial region, which normally gives rise to secondaries and the various types of covert feathers. If, however, portions of skin from the adjacent humeral and tip or hand regions were included, feathers characteristic of these regions always developed. Occasionally, it was possible to remove intact and to transplant nearly all of the wing skin, thereby obtaining all of the many types of wing feathers from one graft. Since wing feathers developed without exception and can be distinguished so easily from the surrounding back feathers, it was not necessary to tattoo or mark the boundaries of the graft in any way.

The successful grafts produced feathers in the same normal succession as the control wing: down, juvenile contour, adult contour. The chief difference in the two sets of contour feathers is one of size, the adult being larger. Mention should be made of an irregularity which often occurred in the graft-plumage sequence. The early-appearing juvenile flight feathers (remiges), which by the time of hatching had either grown sufficiently to emerge from the mouth of the follicle or were nearly ready to emerge, very rarely continued their growth after transplantation to the back of the second host but were shed in this pin-feather stage during the first week, when the transplant was becoming incorporated in its new site. After 2–4 weeks the next generation of feathers, the first adult flight feathers, began to appear from these follicles. Thus, portions of the transplant were producing adult plumage at the same time that other portions and the host body skin in general were producing juvenile feathers (Pl. I, Fig. 6). During the time that the large feathers of the wing type were emerging from the grafted skin, it was necessary to protect the hosts by isolation from other birds, in order to prevent the feathers from being pecked off as fast as they were formed. In spite of all precautions, accidents occurred, with the result that by the time some of the hosts reached adulthood the transplant had already produced several generations of adult feathers.

The color and pattern of the contour feathers developing from the graft after retransplantation were strictly of the host type (Pl. II). As soon as connection was established with the surrounding back skin, melanoblasts of host origin began their migration into the graft, where they laid down in the developing feathers a color pattern indistinguishable from that of homologous feathers of the host wing. This result was consistent whether the transplant was returned to the original donor, as in Series 1, or to other individuals of the same or of a different breed, as in Series 2. When the donor and the host represent two breeds whose feathers show an entirely different color and pattern, such as the Barred Plymouth Rocks and New Hampshire Reds of Series 2, the results show very strikingly that the genotypic composition of the melanophore is the controlling factor in both color and pattern. For example, melanophores from the back region of a Barred Plymouth Rock host produced as perfect and as characteristic a black-and-white barred pattern in the New Hampshire Red wing feathers growing on the back as in homologous feathers growing on the host's own wing. The broad, widely spaced, somewhat irregular black and white bars of the remiges, the narrow, more distinct bars of the smaller coverts, and the variety of in-between types comprising the wing plumage of a normal Barred Plymouth Rock were all faithfully reproduced in corresponding feathers of the grafts of New Hampshire Red origin (see all of the

barred feathers of Pl. II). Likewise, melanophores from the back of New Hampshire Red hosts produced in the wing feathers of the grafts from Barred Rock donors a typical red or a combination of red and black, nonbarred pattern, characteristic of the wing feathers of the Red host breed (Pl. II, Figs. 15, 16, 18, 21, 23).

In the two above-mentioned breeds the color pattern of the back and the wing feathers is sufficiently varied to be readily distinguishable. In the flight feathers and larger coverts of the Barred Plymouth Rock the width of the black and white bands or bars provides the basis for diagnosis. In the New Hampshire Red breed many of the wing feathers exhibit solid black coloration in varying amounts—a coloration which never occurs in feathers of the back. We have noted, already, that the wing feathers of the graft-area growing on the host back always exhibited the characteristic wing pattern of the host, although the melanophores responsible had come in from the surrounding back skin and would have produced a back pattern if they had reached a back-feather follicle. It is evident, therefore, that the feather germ determines, to a large extent, its own specific type of pattern and can modify greatly the details of the pattern produced by the melanophore (cf. Willier, 1941, 1942).

The one F_1 hybrid to which New Hampshire Red skin was retransplanted was a female and turned out to be one of the all-black plumage types rather than one of the more interesting black-red types which frequently show up in the females of this cross (Barred Plymouth Rock female × New Hampshire Red male). Therefore, no differences in pattern between the wing and back feathers were discernible. The wing feathers of the graft showed the typical solid-black host coloration (Pl. II, Fig. 12) with an occasional reddish-brown shaft.

It was mentioned earlier that the donor embryos were, in most cases, allowed to continue their development in order to determine the sex of the grafted skin. In 4 of the 5 successful cases in which newly hatched Barred Rock host chicks received transplants of skin bearing white feathers of New Hampshire Red origin, the sex of the donor was known. The combinations were the following: New Hampshire Red female to Barred Rock male, 2 cases; New Hampshire Red female to Barred Rock female, 1 case; New Hampshire Red male to Barred Rock male, 1 case. In every instance the type of barring produced by the host melanophores in the feathers of the graft, irrespective of the sex of the donor, followed in all exactness the details of tone and pattern characteristic of the sex of the host (for example, the greater width and intensity of the black bars of the female as contrasted with the male).

Precisely the same results were obtained from a series of retransplantations of wing skin from Barred Rock donors to other Barred Rock host chicks. In 6 cases (4 females; 2 males) in which the feathers of the graft were particularly suitable for pattern analysis and persisted long enough to be followed through maturity, the barred pattern produced in the feathers of the graft by the host melanophores was always indistinguishable from that of corresponding feathers of the host wing. As it turned out, in all 6 of these cases the donors died early, before their sex could be recognized with certainty. It is highly probable, however, that some of the combinations were heterosexual.

The results of previous experiments (Willier and Rawles, 1940, 1943) in which melanophores from Barred Plymouth Rock embryos and from a cross

(Barred Plymouth Rock female × Rhode Island Red male) showing distinct sex-linked plumage differences were grafted to the wing bud of embryos of another breed have demonstrated conclusively that the melanophore is the controlling factor in the expression of sex-linked differences in plumage pattern. Also, Danforth's (1929*b*) observations of many years ago on skin transplants made at hatching between individuals of a cross showing sex-linked plumage characteristics pointed to the same conclusion.

The present experiments constitute a further demonstration of the control of barred-pattern dimorphism by the melanophore. It has been seen that the type of barred pattern produced in the pigment-free feathers of the grafts of New Hampshire Red and Barred Plymouth Rock origin corresponded, in all exactness, with that characteristic of the sex of the host which furnished the melanophores. For example, melanophores from a male Barred Plymouth Rock host produced a typical male barred pattern in the grafted feathers of New Hampshire Red origin, regardless of the sex of the donor. Similarly, melanophores from a female Barred Plymouth Rock host produced a female barred pattern in the grafted feathers irrespective of their origin or sex. That the host furnishes both the melanophores and the sex hormones in the design of present experiments is of no consequence, in view of the well-known fact that the dimorphism of the barred pattern is independent of sex hormones, as evidenced by the absence of any modification in the quality of the barring following castration (Montalenti, 1934). The sexual dimorphism exhibited in the barred pattern of the Barred Plymouth Rock is purely of a genetical nature.

The wing is not the ideal region for observing secondary sexual characteristics of the plumage, since the display of such characteristics is more or less confined to the minor coverts of the upper wing surface. It should be added, however, for the sake of completeness, that in the present experiments, where the individuals concerned represented breeds in which the males are normally cock-feathered, the covert feathers of the graft at maturity, regardless of the sex of the donor, exhibited the secondary sexual characteristics of corresponding feathers of the host (Pl. II, Fig. 18).

So far, we have been concerned primarily with those cases in which both the donor and the second host belonged to colored breeds. It will be seen from Table 1 that in a few cases skin from a white donor (White Leghorn) was retransplanted, after differentiation as usual in the White Leghorn coelom, to a pigmented host (Barred Rock), and vice versa. Melanophores from the Barred Rock host entered the melanophore-free White Leghorn feather germs of the grafted wing skin and produced in the developing feathers their own specific type of barred pattern, just as in the pigment-free feathers of the pigmented breeds tested. In the reciprocal experiment, in which the White Leghorn was the second host, the principle involved remains the same. White Leghorn melanophores migrated after hatching into the "white" Barred Rock graft but, true to type, did not deposit pigment granules in the barb and barbule cells of the developing feathers. Therefore, the contour feathers which developed from the graft remained unpigmented. The presence of White Leghorn melanophores in the grafted Barred Rock feathers can be demonstrated easily by making split preparations (Lillie and Juhn, 1932) of the developing feathers from the graft area. The fact that the Barred Rock graft continued to produce unpigmented

contour feathers for as long as the transplant persisted (well over 2 months) would seem strong evidence against any theory of *in situ* pigment formation with respect to feather coloration.

Certain deviations from the usual results described in the preceding paragraphs have occurred from time to time and should be mentioned. Feathers structurally aberrant have been surprisingly few and, without exception, have been confined to the large feathers —i.e., remiges and major coverts. The abnormalities have shown up in several forms, such as much twisted, unexpanded feathers; half-feathers composed of half of the shaft with barbs on one side only or, more rarely, two half-feathers from the same follicle; and feathers with normal shafts but abnormal, partly expanded barbs. Since these types were found at the graft margin, they are the result, in all probability, of mechanical injury to the feather germ at the time the retransplantion was made. Another defect, somewhat minor but of more frequent occurrence, was a shortened calamus with a bent, hooklike tip. Such feathers were normal in other respects. No feathers which could possibly be interpreted as back-wing chimerae were observed along the line of union of the host (back) and graft (wing) skin. The possibility of uniting portions of two feather germs to produce structural mosaic feathers by sewing together incised areas of skin was tested by appropriately designed experiments long ago by Professor Danforth, with wholly negative results (Danforth, 1929*a*, 1939, and personal communication). In view of his wide experience and extensive observations on skin grafting and the results of the present experiments, it seems highly improbable (contrary to the recent suggestion of Wang, 1943, p. 346) that structural mosaics can be obtained by this method. The role of migratory pigment-forming cells (melanoblasts) in the control of color patterns in feathers has been so completely demonstrated in recent years that any attempt to explain the color patterns of mosaic feathers on a purely structural basis seems unnecessary as well as unpromising.

Deviations have occurred more frequently in pigmentation than in structure. Certain feathers—relatively few in number and not confined to any particular region of the graft—have remained white, i.e., have not become invaded by host melanoblasts from the surrounding back skin. Their appearance is much more frequent among feathers emerging early after retransplantation than later on. While some feather germs have continued to produce these exceptional all-white feathers throughout successive feather generations, many have produced feathers with the normal host coloration after the first one or two generations. Once a feather germ has produced a fully pigmented feather, no reversion to the unpigmented type has been observed

Since the transition from white to host color can occur at any stage in the development of the feather, this leads to the formation of a wide variety of mosaics with a variable amount of white in the apical portion of the feather and with the remainder colored like the host. The boundaries between the two regions may or may not be sharply defined. The replacement feathers have usually been host colored.

It should be pointed out that the appearance of the exceptional, all-white contour feathers is correlated in no way with the presence of pigment rings (White Leghorn melanophores) seen at the bases of some of the feathers when the transplants were removed from the White Leghorn coelom (see p. 169). Down feathers with pigment rings were

replaced by contour feathers showing typical host coloration after retransplantation just as freely as were those without rings. An examination of split preparations from many of these exceptional feathers has shown that their "whiteness" is the result of a complete absence of melanophores.

DISCUSSION

The data presented in the preceding pages have demonstrated very clearly that melanoblasts from a newly hatched host migrate readily into the juvenile and adult feathers developing from a graft of wing skin deprived of its normal source of melanophores. We have seen, further, that the color pattern produced in the feathers of the graft by these invading host melanophores is always indistinguishable from that of homologous feathers of the host, regardless of whether the transplants were autoplastic or heteroplastic. When these results are compared with the results of our earlier experiments (Willier and Rawles, 1940), where melanoblasts from donor embryos of one breed were introduced into the base of the developing wing buds of embryos of another breed, we see, at once, striking similarities as regards the migratory capacity and the breed specificity of the melanophore. In both types of experiment, melanophores of one genetic constitution were combined with feather germs of another genetic constitution. In the earlier experiments the tiny piece of embryonic donor tissue supplied the melanoblasts, the host the melanophore-free feather germs, and the invasion occurred during embryonic life. In the present experiments the melanoblasts were supplied by the host, the melanophore-free feather germs by the donor, and invasion occurred during postembryonic life. Irrespective of the differences in donor-host combination and the time when the combination was made, the results have been consistent in producing feathers with coloration like that of the breed which furnished the melanophores.

The only real difference in the results of the two sets of experiments centers around the permanence of the pigmentation effect. Under the conditions of the earlier experiments the donor coloration was not retained permanently, as in the present series, apparently because the original implant of donor tissue did not supply melanoblasts in sufficient quantity to provide the successive generations of feathers with the necessary new complement of melanophores. Consequently, later on, the role of pigmentation was taken over by the melanophores of the host. Recent experiments, soon to be published, indicate that host-donor incompatibility is not involved and appear to verify the suggestion made earlier (Willier and Rawles, 1940, p. 190) that the replacement of donor melanophores by those belonging to the host is due to the introduction of an inadequate quantity of donor melanophore precursors.

The extensive migration of melanoblasts observed in the experiments just mentioned did not occur when skin with its full complement of melanophores was transplanted between two normal, newly hatched chicks of different breeds (Danforth and Foster, 1929; Danforth, 1929*a*). In these experiments the feathers regenerated by the graft possessed the coloration of the donor breed. Sometimes, however, there appeared along the line of union of host and donor tissues a few feathers showing both donor and host pigmentation in mosaic pattern (Danforth, 1929*a* 1935, 1939). On the whole, however, the experimental conditions are not conducive to a migration of host melanophores from the surrounding skin. Why an area of grafted skin

containing its normal supply of melanoblasts blocks the entrance of other melanoblasts while a similar area of melanophore-free skin is so extremely receptive is an interesting question and implies that the melanophore content of the tissue is somehow rather precisely controlled. If melanoblasts were free to migrate at random, one would expect to find a mixture of donor and host coloration in the feathers rather than the observed clear-cut segregation of the two types. Even in the few mosaic feathers at the graft margin there is no evidence of an indiscriminate mixture of the melanophores. Each breed-type usually occupies a separate and distinct area of the feather epidermis. The nature of the mechanism involved in controlling melanoblast migration so effectively in these cases is at present obscure.

Recently Foulks (1943) has been able to show that the feather epidermis of each successive generation of feathers acquires pigment cells as a result of its penetration by melanoblasts from the dermis during the process of regeneration. On the basis of his findings, both histological and experimental, he has postulated a permanent supply or "reservoir" of melanophore stem cells within the dermal tissue external to the feather germ, rather than in the germ itself, as heretofore more or less taken for granted.

On this basis, then, we may assume that in Danforth's cases the area of transplanted normal skin has already acquired during embryonic development a sufficient "reservoir" of potential pigment cells to furnish the feather germs which it contains with a permanent source of melanophores of its own breed-type. In the present experiments no such "reservoir" could be set up during embryonic life, owing to the design of the experiment (early isolation of the wing bud and its subsequent development in the White Leghorn coelom). Later, however, when contact was established with the host skin after hatching, an abundant supply of melanoblasts became available, and a permanent source of host melanophore precursors was then apparently set up in the grafted skin which proved capable of supplying the succeeding generations of feathers with host pigment.

Using an entirely different method, Wang (1941) found that the feathers which regenerated from whole adult papillae (feather germs) transplanted between different breeds of fowl also received their entire supply of pigment from the host. In his experiments and the present experiments, host melanoblasts have penetrated the epidermis of the transplanted feather germs during a relatively late period in development, when juvenile and adult feathers are forming.

So far, all the available evidence indicates that there is no time limit to these events. In the experiments in which melanoblasts were introduced into the wing bud, the replacement of donor melanophores by those of host origin has been observed during the formation of the first set of adult feathers; and in some cases the exceptional white feathers of the present experiments have received a complement of pigment cells from the host after maturity. Also, the variation in the proportions of host pigmentation found in the posterior portion of the mosaic feathers mentioned previously shows that host melanophores can invade the feather epidermis at almost any time during the formation of the feather. In this respect, contour feathers are entirely different from down feathers. Watterson (1942) found that down feathers received melanoblasts from the surrounding skin only at a very limited time in development, just before the onset of feather-germ formation (be-

tween 80 and 91 hours in the case of the wing).

While much progress has been made in recent years concerning the origin of pigment-forming cells, their migratory activities, and their role within the feather follicle, there is still no conclusive information as to their location and distribution in the skin outside of the follicle or their method of getting into the follicle itself. Are they transported entirely along the vascular channels, as suggested by Champy (1935) and Foulks (1943), or can they move directly through the connective tissue of the dermis? The appearance of exceptional, pure-white feathers among the pigmented feathers of the graft-area in the present experiments indicates that melanoblasts do not necessarily follow all of the vascular channels. There can be no doubt that these feathers are well vascularized. Their morphology is perfectly normal in every respect. In the majority of cases the feather germs which gave rise to these unpigmented feathers at first have, later on, received host melanoblasts, as evidenced by the regeneration of feathers completely pigmented. In a few cases, however, melanoblasts have been kept out permanently, and the successive generations of feathers have continued to be white. Just what the nature of the barrier is, is not known. In all probability it is purely mechanical. Abnormal tension brought about by the healing of the skin in its new site, slight adhesions, fiber formation, etc., might easily bring about a rearrangement of connective-tissue cells which could block the paths of melanoblast migration in limited areas for a short time or even permanently. Ris (1941) considers the possibility of mechanical hindrances to pigment-cell migration. He finds, for example, that a sudden change in the arrangement of mesenchyme cells in two adjacent tissues is sufficient to block melanoblast entry. Fast-growing regions, such as limb buds, are particularly favorable for migration, as we have seen from the extensive and rapid spread of pigment occurring when melanoblasts were introduced into the base of the wing bud (Willier and Rawles, 1940). Further, the inherent tendency of melanophores to follow the line of least resistance, as it were, and distribute themselves in abundance along the walls of blood vessels, nerves, sex ducts, etc., when grafts containing precursor melanophores are placed in the White Leghorn coelom, has been pointed out previously by both Ris (1941) and the writer (1940).

Apparently, the paths of migration of the melanoblasts concerned with the normal pigmentation of feathers are restricted to the skin proper. Evidence pertaining to this point has been obtained from an experiment, not mentioned heretofore, carried out in collaboration with Professor Danforth during a visit in his laboratory. A relatively small graft of melanophore-free wing skin, obtained by the usual method, from a Barred Plymouth Rock donor was pushed underneath the intact back skin of the newly hatched donor chick through an incision made a short distance away from the site. The graft was spread out carefully, so that its under surface was in contact with the subdermal connective tissue and muscle of the back of the host. The graft established connection with these tissues and began to produce feathers, which pushed up against the overlying host skin. To relieve the increasing tension on the host skin and to release the outgrowing feathers, an opening was made in the center of the skin growing over the graft. The contour feathers which grew out were all white. Several weeks later the host skin above the graft was trimmed away fur-

ther, so that its edges came in contact with the edges of the graft. At the same time, some of the all-white feathers were plucked from the graft region. The interesting thing is that the feathers which regenerated after contact was established with the skin of the host were pigmented like the feathers of the host, demonstrating that host melanoblasts had entered the grafted feather germs. Besides furnishing information on the location of the paths of migration, this experiment is of further interest in showing that melanoblasts migrate as readily into melanophore-free skin nearing maturity as into the younger skin of newly hatched chicks.

Finally, it is perhaps worth pointing out that, apart from their bearing on pigmentation problems, the experiments reported in this paper are of interest from a purely developmental point of view in demonstrating the complete self-differentiation capacity of the wing bud. At the time the primordium was removed, about the third day of incubation, its wing qualities are determined not only for complete morphogenesis and axiation, as pointed out by Hamburger (1939), but also for the complete development of specific wing feathers arranged in definite tracts. When it is recalled that feather germs do not appear on the wing until the ninth day of incubation—approximately 6 days after the wing bud was isolated—the minuteness with which the feathers of the transplanted wing skin growing on the back agree in detail with corresponding feathers from the donor wing is, indeed, striking.

SUMMARY

1. Pigment-free skin and feathers were obtained by removing the right wing bud from donor embryos of pigmented breeds (New Hampshire Red and Barred Plymouth Rock, chiefly) before the neural-crest cells had migrated into it and by allowing it to continue its development in the coelom of the White Leghorn host embryo.

2. At hatching, or soon afterward, a piece of skin with its white plumage (down plus the primordia of the juvenile flight feathers) was dissected from the intracoelomic wing graft and retransplanted to the back region of a newly hatched chick (host No. 2) of a pigmented breed (Barred Plymouth Rock, New Hampshire Red, F_1 hybrid).

3. The retransplantations were of two types: (1) those to the original donor chick (autoplastic) and (2) those to normal chicks of the same breed (homoplastic) or of a different breed (heteroplastic). The results of the two series were in complete agreement with the exception of tissue antagonism or incompatibility, which occurred frequently when chicks other than the original donors were used for the second hosts.

4. Without exception, the successful grafts produced wing contour feathers, in normal succession, typical of the portion of the wing from which the skin was taken.

5. The color and pattern of the feathers of the graft were strictly of the host type. When connection was established with the surrounding back skin, melanoblasts of host origin migrated into the graft and laid down in the developing feathers, regardless of their origin, a color pattern indistinguishable from that of homologous feathers of the host wing.

6. When donor and host represent two breeds whose feathers show an entirely different color and pattern—e.g., Barred Plymouth Rock and New Hampshire Red—the results show very strikingly

that the genotypic composition of the melanophore is the controlling factor in both color and pattern.

7. Since melanophores from the same region—the back—of the host always produced a typical wing pattern in the wing feathers of the graft, and at the same time a typical back pattern in the adjacent back feathers of the host, it is evident that the epidermis of the feather germ determines, to a large extent, its own specific type of pattern and can modify the details of the pattern produced by the melanophore.

8. Exceptional feathers, relatively few in number and not confined to any particular region of the graft, have remained unpigmented (white) for varying periods of time and even permanently. The nature of the barrier to host melanophore entrance is probably purely mechanical. Once a transplanted feather germ has produced a fully pigmented feather, no reversion to the unpigmented type has been observed.

9. The results of the present experiments have demonstrated clearly that host melanoblasts will readily enter the regenerating feathers of a grafted area of skin deprived of its normal complement of melanophores. The fact that a similar migration did not occur when the skin graft was fully pigmented at the time of transplantation (Danforth, *loc. cit.*) implies that the melanophore content of the skin is somehow rather precisely controlled. The nature of the control is at present obscure.

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PLATE I

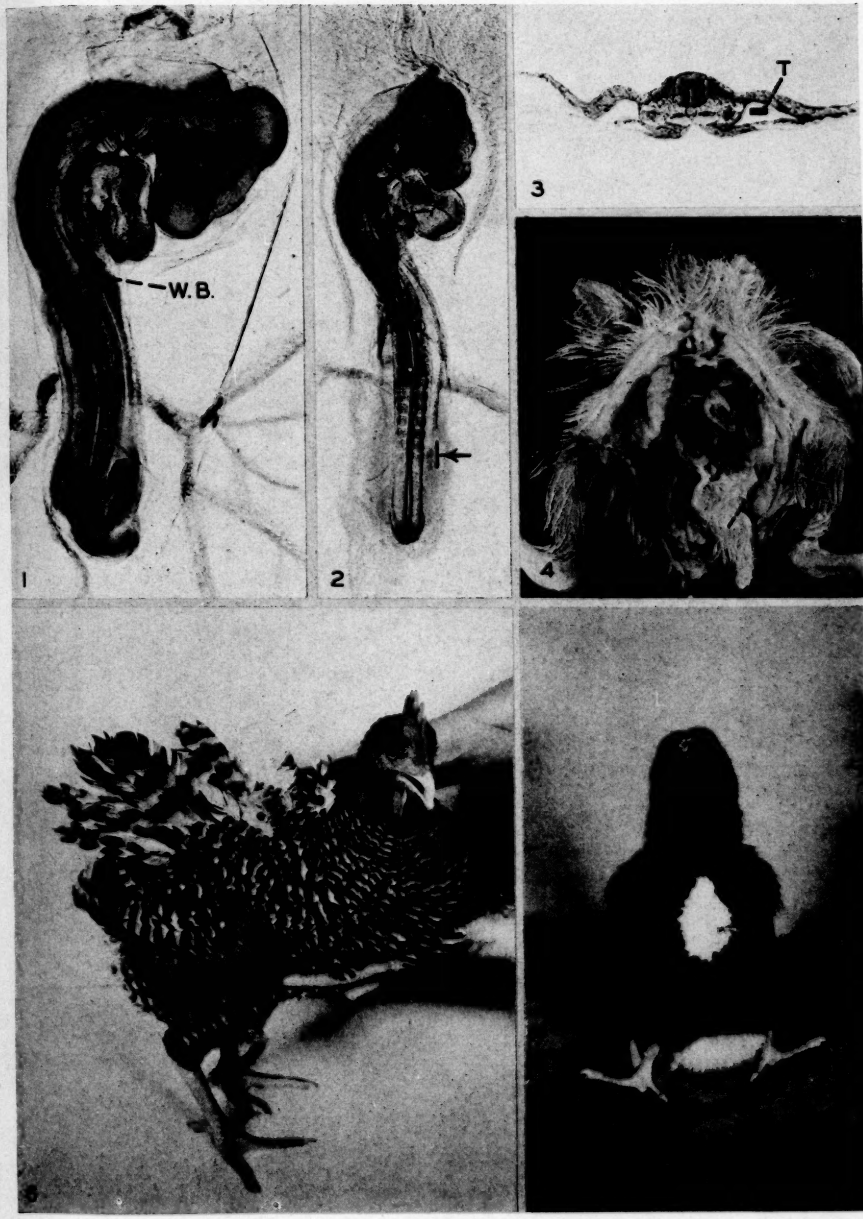

PLATE II

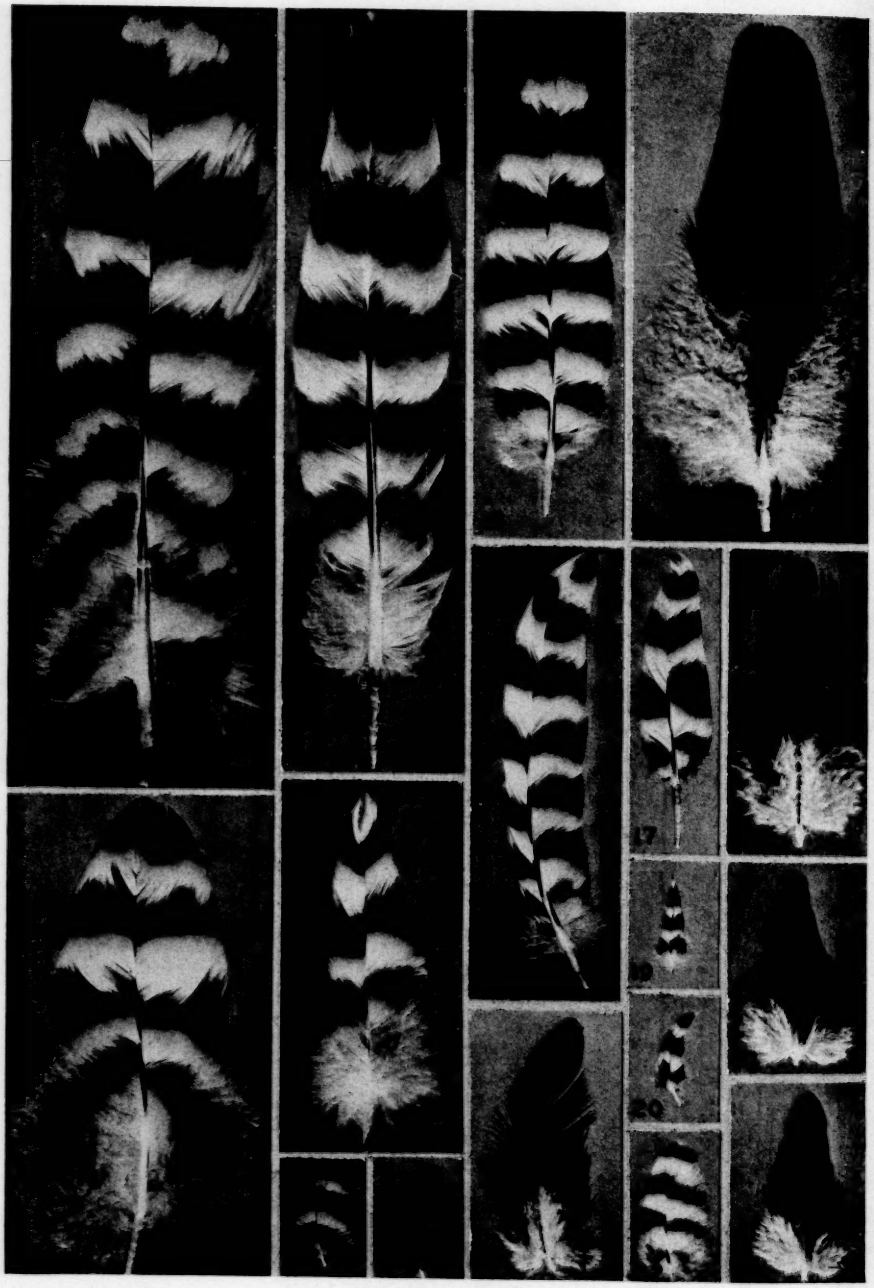

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PLATE I

A series of photographs illustrating the various steps involved in obtaining pigment-free wing skin from a New Hampshire Red donor and its retransplantation to the back of a Barred Plymouth Rock host chick.

FIG. 1.—New Hampshire Red donor embryo (72 hours) showing the right wing bud (*W.B.*) at the time of its removal. ×12.

FIG. 2.—White Leghorn embryo of 60 hours (host No. 1). Arrow points to slit in somatopleure through which the wing bud (*W.B.* of Fig. 1) was introduced into the coelom. ×12.

FIG. 3.—Cross-section through White Leghorn host embryo of Fig. 2 at level where graft was introduced into coelom. Note transplant *T* (diagrammatically represented) lying in the coelom. ×40.

FIG. 4.—White Leghorn host embryo of Fig. 2 at hatching. Body cavity exposed to show the wing graft (*W*) bearing white (pigment-free) plumage.

FIG. 5.—Barred Plymouth Rock chick (host No. 2). White area on back is retransplanted wing skin, bearing white down, dissected from intracoelomic wing graft (*W*) shown in Fig. 4. Photographed 1 week after retransplantation.

FIG. 6.—Barred Plymouth Rock male (host No. 2) three months after retransplantation of pigment-free wing skin of New Hampshire Red (female) origin.

Note that the white down has been replaced by typical wing contour plumage showing the black and white barred pattern characteristic of homologous feathers of the host wing. (Flight feathers represent first adult series. See text.)

PLATE II

A series of photographs at the same magnification (×¾) illustrating the color and pattern of some of the various types of wing feathers produced by grafts of pigment-free wing skin grown on the back of pigmented hosts of a different breed.

FIGS. 7, 8, 10, 13.—Flight feather (secondary) and three types of covert feathers regenerated during third year. Note typical black-and-white barred pattern produced by melanophores from a Barred Rock male host. Skin from New Hampshire Red female donor.

FIGS. 9, 14, 17, 19, 20, 22.—Flight feathers and coverts from the bird shown in Pl. I, Fig. 6.

FIG. 11.—A small upper covert feather showing the normal Barred Plymoth Rock pattern of the female host. Skin from New Hampshire Red female donor.

FIG. 12.—Upper covert feather, regenerated during third year, showing the typical solid black coloration of an all-black female hybrid host. Skin from New Hampshire Red donor.

FIGS. 15, 16, 18, 21, 23.—Covert feathers showing both the solid red coloration (Figs. 18, 21) and a combination of red and black coloration (Figs. 15, 16, 23) characteristic of homologous feathers of the New Hampshire Red male host wing. Skin from Barred Plymouth Rock female donor. Note the fringing (male secondary sexual characteristic) of Fig. 18.

EFFECT OF OXYGEN CONCENTRATION ON THE DEVELOPMENT OF THE CHICK EMBRYO

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CONSIDERABLE work has been done on the general respiration of the embryo; but it is surprising to find that research dealing with the effect of oxygen on the development of the incubating egg is very meager, especially the effect of oxygen concentration higher than atmospheric.

From earlier investigations Preyer (1885) concluded that a proper supply of oxygen is more essential for growth than for differentiation. Wesselkin (1913) found that low oxygen content does not permit the embryo to survive long. On a few eggs he showed that the embryo can survive about 48 hours in 5 per cent, and 72 hours in 12 per cent, oxygen. The dove embryo probably requires not less than 14 per cent oxygen for the whole period of incubation, according to Riddle (1924), who also suggested that low oxygen concentration is more fatal to the older, than to the younger, embryo and that high oxygen concentration has the opposite effect.

The only work on the effect of different oxygen concentrations below and above 21 per cent is that of Barott (1937). From the incubation of eggs exposed to oxygen concentrations of 15, 18, 21, 30, 40, and 50 per cent continuously from the first day up to hatching, he found that the highest hatchability can be obtained at a concentration of 21 per cent oxygen.

The effect of oxygen concentration, in the early stages, on the growth and development of the embryo and on consequent survival and hatchability of eggs still remained to be determined. So this study was undertaken. The results may throw more light on the effects of gaseous environment on the avian embryo and add to the general knowledge of physiological embryology.

MATERIAL AND METHODS

About eight hundred fertile fresh eggs of White Leghorns laid during the fall months were exposed to various concentrations of oxygen, ranging from 11 to 95 per cent. For this purpose, several glass vacuum desiccators, each having a capacity of about 7 liters, were used. The desired concentration of oxygen was obtained by the fractional evacuation of air from the desiccator and then by refilling it with pure oxygen or pure nitrogen. After the adjustment of the gaseous composition, the sealed desiccators, with eggs, were placed in a constant-temperature room at 37.5° C. The desiccators were tilted alternately, right and left, about 45° at 8-hour intervals.

Preliminary tests had indicated that eighteen fertile eggs placed in such a sealed desiccator, with the initial oxygen concentration of about 21 per cent, were able to develop for the first 5 days and that, if thereafter incubated under ordinary conditions (temperature 37.5° C. and relative humidity about 60 per cent), they were able to hatch. It seems that during this 5-day period the reduction of nearly 2 per cent in oxygen and the increase of about 2 per cent in carbon dioxide concentration in the sealed egg container, as determined by gas analysis, did not interefere seriously with the course of embryonic development.

Consequently, the 5-day period of exposure of fresh eggs was adopted in the present study. In the observations on the early growth of the embryo, however, the eggs were first incubated 60 hours under normal conditions; and their fertility was determined by means of transmitted light (or candling), previous to exposure.

EXPERIMENTAL RESULTS

Mortality and tolerance to exposure.—The mortality of the embryo during the 5-day exposure was least within the range of about 15–50 per cent of oxygen concentration. Below this concentration there was an increase in mortality, and above 65 per cent oxygen concentration the mortality was much greater. Above 75 per cent it was very high. This indicates that abnormally high oxygen concentrations are harmful to the young embryo.

Growth of the embryo.—During the first 5 days of exposure the growth of the embryo was most rapid, with 41 per cent oxygen (at 5 days the embryo weighed, on an average, 0.089 ± 0.006 gm.). With 21 per cent oxygen, the embryo weighed 0.051 ± 0.007 gm.; with 31 per cent, 0.071 ± 0.003 gm.; with 51 per cent, 0.071 ± 0.002 gm.; with 61 per cent, 0.068 ± 0.010 gm.; and with 71 per cent, 0.056 ± 0.014 gm.

After 60 hours of preliminary incubation under normal conditions, the eggs exposed to 21, 31, and 41 per cent oxygen for the periods of from 2 to 6 days reveal further interesting facts. At this stage the growth of the embryo (Table 1) was more rapid at 31 per cent oxygen concentration. But, when the oxygen concentration was raised to 41 per cent, the growth of the embryo, although still

TABLE 1

THE EFFECT OF 2, 4, AND 6 DAYS OF EXPOSURE TO 21, 31, AND 41 PER CENT OXYGEN CONCENTRATION ON THE GROWTH OF THE CHICK EMBRYO AFTER 60 HOURS OF NORMAL INCUBATION

Periods of Exposure (Days)	Relative Weight of Embryo*					
	21 Per Cent Oxygen		31 Per Cent Oxygen		41 Per Cent Oxygen	
	Grams	Per Cent	Grams	Per Cent	Grams	Per Cent
2	0.074±0.014†	100.0	0.150±0.030	202.7	0.133±0.035	179.7
4	0.420±0.058	100.0	0.683±0.040	162.6	0.550±0.074	131.0
6	1.148±0.045	100.0	1.645±0.118	143.3	1.593±0.079	138.8

* Each group represents four typical embryos.

† Standard deviation after the formula $\delta = \sqrt{[\Sigma(x^2)]/(N-1)}$, where x is the deviation from the arithmetic mean and N is the total number of observations.

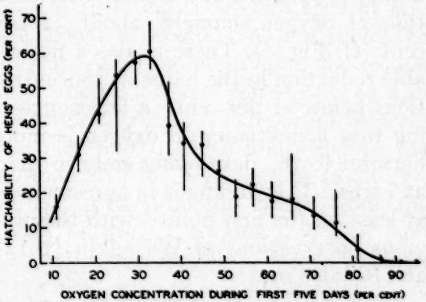

FIG. 1.—The effect of different concentrations of oxygen during the first 5 days of incubation on hatchability of hens' eggs. Based on hatchings at twenty different conditions. Vertical lines show actual deviations of two or more experiments at each condition.

much greater than at 21 per cent, was slowed down.

Hatchability of eggs.—Figure 1 shows the effect of exposure of the incubating eggs in groups of eighteen to different concentrations of oxygen on hatchability. It may be noted that the highest hatchability was obtained when the initial content of oxygen was 32 per cent. It is also evident that, although the embryo can survive a 5-day exposure at concentration below 21 per cent, hatchability is reduced markedly at low concentrations of oxygen.

DISCUSSION

The most interesting fact revealed by this study is that the avian embryo during early stages responds to concentrations of oxygen greater than 21 per cent. Barott's (1937) experiments on the continuous exposure to different concentrations of oxygen gave the highest hatchability of hen's egg at normal concentration (21 per cent). Our results (in spite of the fact that the eggs were incubated during unnatural hatching season—in the fall) on the exposures during the first 5 days of embryonic life indicate that the maximum hatchability is obtained at a higher concentration of oxygen, namely, about 32 per cent (cf. Fig. 1). There is also a noticeable reduction in the hatch at concentrations below 21 per cent—a fact suggesting that a deficiency in oxygen is more harmful to the developing embryo than an excess. This finding is in agreement—at least on the first point—with the previous observations of Wesselkin (1913) and Riddle (1924).

Since a concentration of oxygen of about 30 per cent tends to accelerate the early growth, it would also be of interest to investigate the combined effect of higher temperature, higher humidity, and higher oxygen concentration than normally in use. It has definitely been shown that high temperature (Romanoff, Smith, and Sullivan, 1938) and high humidity (Romanoff, 1929) favor the development of the early embryo. On the other hand, a moderate amount of carbon dioxide (about 0.4 per cent) also stimulates the growth of the embryo during the first few days of incubation (Romanoff, 1930). This would seem again to offer some new possibilities for the work on the oxygen activation, in combination with the other gaseous and physical environments.

SUMMARY

The chick embryo can survive after exposure to an oxygen concentration as low as 11 per cent for 5 days, and some even hatch after exposure if incubated under normal conditions.

The embryo can tolerate for 5 days an oxygen concentration up to 75 per cent. Beyond this concentration the mortality is very high.

The early growth of the embryo is accelerated by exposure to oxygen concentrations above 21 per cent. The highest growth was observed at an initial concentration of about 31–41 per cent.

The hatchability of eggs can be influenced favorably or unfavorably by exposure during the early part of incubation to different oxygen concentrations. The highest hatchability was obtained at an oxygen concentration of about 32 per cent.

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EFFECT OF LIGHT UPON TIME OF OVIPOSITION IN RINGDOVES

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LITTLE is known of the relation of light to periodicity of oviposition in birds. Earlier observations on doves, pigeons, and fowl have indicated, however, that such a relationship really exists. This type of periodicity, unlike that involved in annual breeding cycles and in migration, is apparently not regulated predominantly through output of gonadotrophin by the anterior pituitary. It is, therefore, of interest to record an extension of our information on effects of seasonal changes in light, and of temporarily clear or cloudy sky, on the time of oviposition in ringdoves.

For several species of pigeons, including the ringdoves (*Streptopelia risoria*), described here, Whitman (1919) recorded the exact time at which a few hundreds of eggs were laid at various seasons of the year. He concluded that "first" eggs of the clutch of two were laid mostly before 5:00 P.M. during the short days of winter and mostly after 5:00 P.M. in summer. Also, that "second" eggs were laid later in the morning in association with the late-rising winter sun, but definitely earlier during summer, when the sun rises earlier. Warren and Scott (1936) found that under normal conditions the onset of darkness seems to be a factor in restricting oviposition in hens to the daylight period; this restriction, however, seemed to be much more directly related to the facts that fowls do not ovulate after the early afternoon and that laying occurs only after egg formation is completed by 26 hours spent in the oviduct. Under continuous lighting, hens will lay at any hour of day and night; and after a period of about 5 days they will ovulate and lay only or chiefly at night under reversal of usual lighting periods.

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PROCEDURE

Female ringdoves normally lay the first of their clutch of two eggs in late afternoon (at about 5:00 P.M.) and the second egg on the morning of the second day thereafter, at about 8:45 A.M. (Whitman, 1919). Each ovum is ovulated at about 38–39 hours before the normal time for it to be laid as an egg. "First" ova probably break from the ovary between 1:00 and 3:00 A.M.; and "second" ova usually ovulate between 6:30 and 7:00 P.M. The ovulation of "second" eggs thus occurs about 2 hours after "first" eggs are laid (Riddle, 1923*a*, and unpublished data). The "first" egg (and the "second") can usually be palpated in the oviduct for as much as 33 hours before it is to be laid. In the fore-

noon of each day it is, therefore, a simple matter to learn, by palpation, which ringdoves are to lay a "first" egg on either that afternoon (hard-shelled egg in oviduct) or the following afternoon (shell soft or absent). To determine the time at which "first" eggs were laid in the present study, half-hourly visits to nests began daily at 3:30 P.M. and continued until all anticipated eggs were laid. With rare exceptions, all "first" eggs were laid before 6:00 P.M. during the winter months and before 7:00 P.M. in the late spring and summer. Much previous experience had shown that extremely few "first" eggs are laid before 3:00 P.M. at any season. On the morning of the second day after "first" eggs were laid, those same nests were again examined for "second" eggs at half-hour intervals; this search began at 8:30 A.M. (winter) or 7:30 A.M. (late spring) and continued, when necessary, to 12:00 N. or until the "second" egg was laid.

Occasionally ringdoves fail to ovulate (and lay) a "second" egg, and still more rarely a perfectly formed egg is retained abnormally for several hours or days unless it is removed surgically or by injection of posterior lobe hormone (Riddle, 1921). Cases of the latter type are not included in the present data. While making the records for time of laying, we further observed and recorded the condition of the sky as "clear," "partly cloudy," or "cloudy." This was done in order to obtain quantitative data on a point which seemed already established by long previous experience with this colony of birds—namely, that on cloudy afternoons "first" eggs are laid earlier, and on cloudy mornings "second" eggs are laid later, than when skies are clear.

OBSERVATIONS

The time, to within a half-hour, of laying of 1,604 eggs from November 18 to June 23 was recorded. This term of slightly more than 6 months is divided, for convenience, into five periods. Within the first period, from November 18 to December 24, days are short and becoming shorter; in the four following periods into which our data were arbitrarily subdivided, days are becoming longer. The curves at the bottom of Figure 1 show the number of eggs laid during each half-hour interval as a percentage of the total number of eggs laid in that particular period. Eggs of the "clear," "partly cloudy," and "cloudy" classes are here all grouped together. In all other curves shown in Figure 1, those eggs which were laid when the sky was "partly cloudy" are entirely omitted, and the eggs which were laid in clear and cloudy weather are recorded separately.

The data show that, when days are becoming longer, the "first" egg of the clutch tends to be laid progressively later in the afternoon; and, conversely, the "second" egg is laid progressively earlier in the morning. The half-hour interval in which the greatest number of "first" eggs are laid and the half-hour interval in which the last of "first" eggs are laid both tend to shift progressively to a later hour in the four periods following December 24 (Fig. 1). Although the percentage of eggs laid before 3:30 P.M. decreases as the days become longer, a few eggs are laid before that time in all five periods. The curves which describe the time of laying of "second" eggs are less uniform, but they show that the peak of oviposition tends to shift to an earlier hour of the morning as the sun rises earlier and the days become longer. These curves further suggest that some eggs were laid more than half an hour before the earliest morning examination; also, that the time of the earliest ovipositions is probably progressively earlier as the days become longer. Thus, confirming Whitman (1919), the total time (in-

terval) between the laying of the two eggs of the dove's clutch is notably diminished in summer.

with clear or cloudy sky. Although the type of sky was classified subjectively, it seems obvious that, by neglecting the

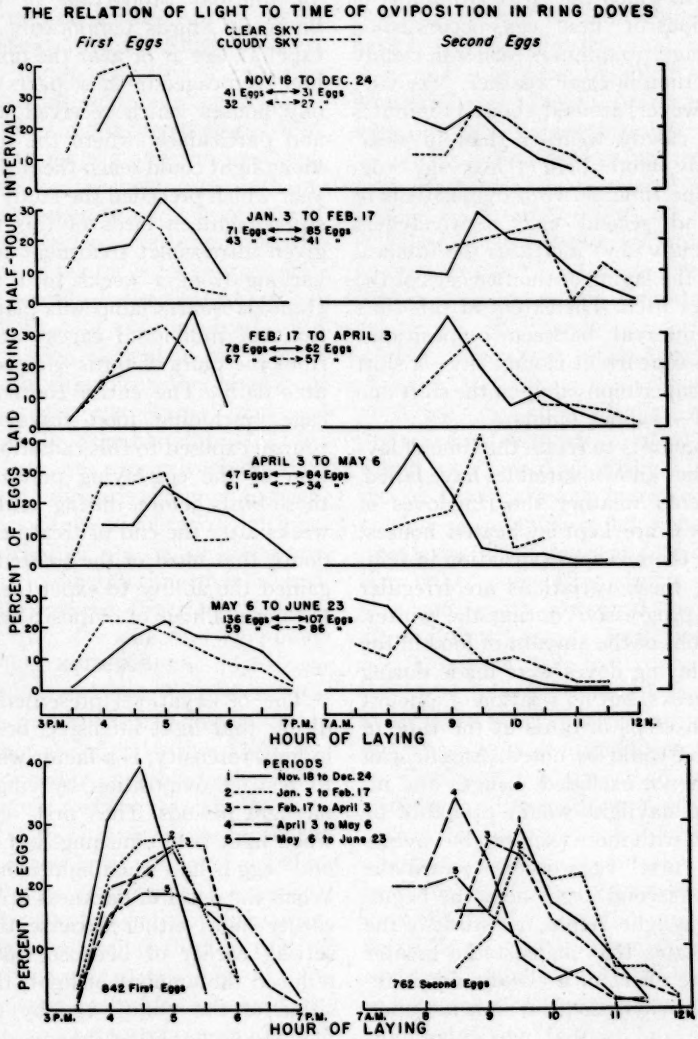
THE RELATION OF LIGHT TO TIME OF OVIPOSITION IN RING DOVES
First Eggs
CLEAR SKY
CLOUDY SKY
Second Eggs
NOV. 18 TO DEC. 24
41 Eggs 31 Eggs
32 " 27 "
JAN. 3 TO FEB. 17
71 Eggs 85 Eggs
43 " 41 "
FEB. 17 TO APRIL 3
78 Eggs 62 Eggs
67 " 57 "
APRIL 3 TO MAY 6
47 Eggs 84 Eggs
61 " 34 "
MAY 6 TO JUNE 23
136 Eggs 107 Eggs
59 " 86 "
PERCENT OF EGGS LAID DURING HALF-HOUR INTERVALS
3P.M. 4 5 6 7P.M. 7A.M. 8 9 10 11 12N.
HOUR OF LAYING
PERIODS
1 Nov. 18 to Dec. 24
2 Jan. 3 to Feb. 17
3 Feb. 17 to April 3
4 April 3 to May 6
5 May 6 to June 23
PERCENT OF EGGS
842 First Eggs
762 Second Eggs
HOUR OF LAYING

FIG. 1

Besides these effects associated with season, i.e., with later sunset and earlier sunrise, the observations likewise show effects of temporary (for the half-day) differences in light intensity associated

data collected on "partly cloudy" half-days, we have data for two fairly distinct degrees of light intensity. The curves describing these data show (all upper curves of Fig. 1) that "first" eggs

tend to be laid sooner, and "second" eggs later, in definitely cloudy weather than in definitely clear weather. The half-hour interval in which the greatest number of ovipositions of "first" eggs occurs is, on the average, 30 minutes earlier in cloudy weather than in clear weather. "Second" eggs, however, are laid about 45 minutes later in cloudy weather than in clear. Relatively more light (clear sky) decreases the time between ovipositions of "first" and "second" eggs; relatively less light (cloudy sky) increases the interval between the laying of the two eggs of the ringdove clutch. The extent of this shift of the interval between ovipositions, owing to concurrent cloudy sky—a shift which is superimposed upon the shift due to season—is about $1\frac{1}{4}$ hours.

Our attempts to relate the time of laying to other known variables have failed. During cold weather the ringdoves of this colony are kept in heated houses; although there is some variation in temperature, these variations are irregular and not progressive during the winter. Estimations of the amount of food in the crops of laying doves were made during several weeks, but no relation of amount of food in crops of birds at the time of oviposition could be noted. An effect of exercise is not excluded. Length and intensity of daylight would probably be associated with more exercise; but oviposition of "first" eggs occurs toward the end, and "second" eggs near the beginning, of daylight. Again, our study of the data indicates that at least the greater part of the effect of a cloudy sky is restricted to oviposition (i.e., it is not upon ovulation) and to that particular half-day on which oviposition actually occurs. Cloudy sky on the day preceding ovulation, or on the first day following ovulation, appears to have little or no effect on the time of oviposition.

Reference should be made to some related observations which cannot readily be treated quantitatively. Over a period of many years it was noted that an unusual proportion of egg-bound birds, i.e., birds temporarily unable to expel an egg at or near the normal time, had been caged in those parts of our colony houses which received least light, and particularly where the least ultra-violet light could reach them. During the year which preceded the study on oviposition eighteen birds of this type were given ultra-violet treatment for periods varying from 2 weeks to 1 month. A Hanovia quartz lamp was placed at the front of individual cages—at 2–5 feet from the pairs of birds—for 20–30 minutes daily. The entire contents of the cage (including food trays) were, of course, exposed to this radiation. Inspection of the egg-laying performance of these birds before, during, and for some weeks after the end of treatment left no doubt that most of the treated birds regained the ability to expel their eggs at the normal hour of oviposition.

DISCUSSION

The observations presented here indicate that light intensity, or a change in light intensity, is a factor which tends to restrict oviposition by ringdoves to daylight periods. The "first" egg is laid when light is diminishing, but the "second" egg is laid when light is increasing. When the onset of darkness occurs at an earlier hour, either because the sun is setting earlier or because clouds have reduced the amount of light, the oviposition of the "first" egg by ringdoves tends to occur earlier than on days when twilight comes at a later hour. Quite conversely, however, the "second" egg of the clutch of two is laid later in the morning when the sun rises later, and also when clouds reduce the amount of sunlight. Chickens, like ringdoves, normally

lay only or chiefly in daylight; but Warren and Scott (1936) showed that under constant artificial illumination hens lay during both day and night. Hens, like ringdoves, ovulate shortly after oviposition (Patterson, 1910). Phillips and Warren (1937) found a positive correlation between the interval between ovipositions and the time intervening between the laying of one egg and the ovulation of what will be the next egg. Both intervals were longer in the winter than in the spring. This longer interval between ovipositions in winter is, of course, similar to that described above for ringdoves.

The present data are concerned solely with the relation of light to the time of oviposition in ringdoves. The results obtained, however, necessarily raise the question as to whether the observed effects of light are narrowly confined to one or a few hours preceding oviposition, and to oviposition alone (oviducal function), or whether season or cloudiness affects the growth and time of dehiscence of the ovum (ovarian function). For purposes of reply, the effect of cloudiness and the effect of season should be considered separately. Both the present data and much additional experience clearly indicate that the effect of the cloudy sky is chiefly or solely upon oviposition; it is thus an action upon or through the oviduct, and there is little reason to believe that the ovary is at all involved. The time shift in oviposition with season is, however, probably a quite different matter, involving a similar shift in time of ovulation. The available data on the time of ovulation in ringdoves do not permit a decision concerning an effect of season; they do indicate a variation of approximately 2 hours at the same season. Levi (1941) presented a photograph and brief description of a pigeon reared to maturity with a congenital absence of the eyeballs. Both first and second eggs were laid by this bird at the normal time of day. Phillips and Warren (1937) think that, in chickens, light does not affect the time of either oviposition or ovulation directly, but these investigators regard it as more probable that its action is upon the growing ovum at about 60 hours before ovulation. An excellent review of data concerning exteroceptive factors in sexual periodicity (pituitary-nervous-ovarian relationships) has been written by Marshall (1942).

Part of a mechanism by which light, acting through the pituitary, may regulate time of ovulation is perhaps suggested by the results of two recent studies. Fraps and Riley (1942) found that hens treated with a preparation of pituitary luteinizing hormone following pretreatment with pregnant mare serum would ovulate. And Fiske (1941) observed that darkness increases the secretion of luteinizing hormone by rat pituitaries. Both doves and pigeons ovulate either soon after darkness begins ("second" eggs) or several hours thereafter ("first" eggs); fowl, as already noted, scatter their ovulations between the early morning and the early afternoon. Thus we are unable, at present, to put these species differences into any common frame. Moreover, though there is an over-all probability that, in doves, light is somehow associated with the seasonal shift in time of ovulation, we must here deal with the paradox that a prolongation of daylight is associated with a delay of the oviposition of the "first" egg and that a prolongation of darkness or light of low intensity is associated with a delay of the oviposition of the "second" egg.

It is certain that both hormonal and psychic factors may either hasten or delay oviposition. Riddle (1921; 1923*b*) showed that posterior lobe hormone (Pituitrin, Pitocin) would cause almost

immediate oviposition of dove and pigeon eggs that were premature by as much as 30 hours, and that it had a similar action in fowl. Burrows and Byerly (1942) have made a further study of this action in fowl. In this connection Jores (1935) has reported—though this was not confirmed by Simon (1936)—that pressor and oxytocic principles, but not intermedin, are much increased in hypophyses (mice, guinea pig) by darkness. Here one might hope to find—but actually we do not find—a basis for periodic release of posterior lobe hormone which would coincide with time of oviposition. Dunham and Riddle (1942) found that injections of desoxycorticosterone acetate and estradiol benzoate, but not estrone and dehydroandrosterone, sometimes delay and sometimes hasten oviposition in ringdoves; and that progesterone tends to delay oviposition. Whether these steroid hormones play a part in normal oviposition over and above the ability of estrogens to develop the oviduct and sustain its motility is not definitely known.

The role of psychic factors inheres in the fact that oviposition is partly a voluntary act. Whitman (1919) noted that the oviposition of "second" eggs is sometimes delayed by the fact that the male has then begun the duties of incubation and may be unwilling to yield the nest when the female needs it. Again, common experience shows that fright or rough handling of doves may cause them to drop their eggs prematurely. The fact that cloudy skies hasten the laying of "first," but delay the laying of "second," eggs probably has a psychological, rather than a hormonal, basis. Oviducal state and motility are predominantly regulated by hormones —its contractions being much exaggerated by posterior pituitary—and it seems probable that posterior lobe hormone action assists in the actual expulsion of the egg; but there is a margin of perhaps an hour or more for the reinforcement or "triggering" of this hormonal preparation by the voluntary act of the bird.

SUMMARY

The time of oviposition of 1,604 eggs by a colony of ringdoves was determined for the period November 18–June 23. As the number of hours of daylight increased from winter to summer, there was a tendency for "first" eggs of the clutch of two to be laid progressively later in the afternoon and, conversely, for "second" eggs to be laid progressively earlier in the morning. This seasonal shift in time of oviposition confirms Whitman and is probably associated with a light-controlled shift in time of ovulation.

At all seasons the time of oviposition is affected by cloudy weather. This effect is independent of time of ovulation. On definitely cloudy days the half-hour interval in which the greatest number of "first" eggs were laid was about 30 minutes earlier, and the similar interval for "second" eggs about 45 minutes later, than on cloudless days.

Attempts to relate the time of laying to variables other than light intensity or change in light intensity were not successful. These observations suggest that light is a factor which tends to restrict oviposition to daylight periods.

Doves which had long occupied cages with restricted lighting were observed to be frequently unable to expel an egg at the normal time for oviposition. Daily ultra-violet radiation of such birds over periods of 2–4 weeks largely corrected this disability.

The exact time of oviposition is certainly influenced by psychological factors, and probably also by posterior lobe hormone.

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THE ELECTRICAL CHANGES IN RESPONSE TO ILLUMINATION OF THE DARK- AND LIGHT-ADAPTED EYE OF DISSOSTEIRA CAROLINA[1]

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THE complex electrical change obtained in response to illumination of the eyes of invertebrates has been interpreted as a summation of several component potentials originating in various regions of the eye and associated nervous structures. From the eye of the squid, Therman (1940) obtained electrical changes upon illumination which he interpreted as being the sum of two component potentials of opposite sign when the two electrodes were placed on opposite sides of the retina. The isolated eye of *Dytiscus* has been used by Bernhard (1942), who interpreted his records as indicating that the receptorial layer of this eye is the origin of a monophasic wave composed of two negative components. He also concluded that the optic ganglion makes a contribution to the electrogram. Recently Jahn and Wulff (1942) have published results on two species of grasshoppers which indicate that both the optic ganglion and the receptorial layer of the compound eye contribute to the wave form of the electrical response. The ganglion, these authors conclude, gives rise to a slow wave that is electrically positive with respect to the corneal lead, while the sensory layer is the origin of a slow negative wave.

Inspection of the records obtained

[1] While this report was in process of completion, the sudden death of Professor I. R. Taylor occurred. The field of physiological zoölogy has lost a valuable contributor; his colleagues and associates will miss a real friend, while students at Brown University will no longer have the benefit of association with a fine gentleman and teacher.—F. C.

from the eyes of invertebrates reveals that the development of a wave or waves indicating negativity of the cornea with respect to more basal regions of the eye upon illumination is a constant and prominent characteristic. In some species the negative condition may be preceded by a positive *a*-wave, which may be quite prominent under certain condi-

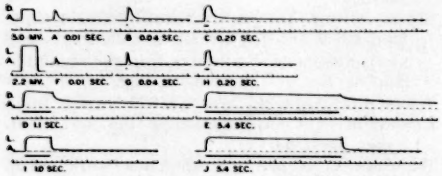

FIG. 1.—Electrical responses of the dark-adapted eye (*D.A.*) and the light-adapted eye (*L.A.*) to exposures of different durations. This series is one showing a high negative value of the illumination potential and a very prominent negative after-potential. It will be noted that light adaptation increases the height of the illumination potential relative to the *b*-wave, enhances the *d*-wave, and markedly reduces the magnitude and duration of the negative after-potential. In this and the succeeding figures the time intervals are 0.2 second, and the duration of illumination of the eye is indicated by a black horizontal line just above the time line. The square-wave calibrations taken for the responses in both the dark-adapted and the light-adapted series are shown. A negative potential of the corneal lead of the illuminated eye is indicated by a deflection upward. To facilitate reproduction, Figs. 1–4, inclusive, were made by photographing tracings constructed from projections of the original records.

tions (Crescitelli and Jahn, 1939; Jahn and Crescitelli, 1939; Taylor and Nickerson, 1943). Hartline (1928), who recorded the electrical changes to illumination of the eyes of several species of arthropods, observed in the grasshopper *Chortophaga viridifasciata* that the initial negative potential declined with continued illumination to a level below the base line, thus indicating the continuous and gradual change from a negative to a definitely positive condition of the corneal region. When this exceptional type of response was detected by the authors in the grasshopper *Dissosteira carolina*, the present study of its details and its change during light adaptation was undertaken. Information enabling comparisons to be made in the electrograms of different species is not only of interest in itself but may also be useful in an analysis of the fundamental nature of the complex electrical response of the eye upon illumination.

EXPERIMENTAL PROCEDURE

In the present experiments the electrical responses from twenty grasshoppers

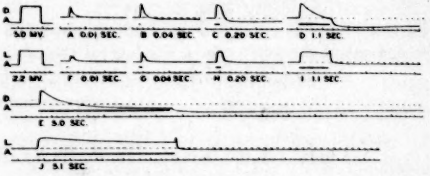

FIG. 2.—Responses of the dark-adapted and light-adapted eyes to exposures of different durations. This series is one showing a relatively rapid decline of the illumination potential during long exposures of the dark-adapted eye. A well-developed, positive after-potential is also shown here for the dark-adapted eye. The double peak in the response of the light-adapted eye to brief exposures will be noted (records *F* and *G*).

were recorded, employing a technique similar to that already described (Jahn and Crescitelli, 1938). Leads were taken from both intact eyes by means of silver–silver chloride electrodes, with Belâr's solution making electrical contact with the corneal surface. One eye was illuminated as desired, while the second eye, kept in the dark, served as the "indifferent" lead. A 500-watt projection lamp (110 volts, D.C.), giving an illumination of 290 foot-candles at the eye, served as a source of light. A sectored-disk shutter, driven by a synchronous motor, was employed to give brief exposures, while a hand-operated photographic shutter served for the longer periods of illumina-

tion. The potentials were amplified by means of a Forbes and Grass (1937) direct-coupled amplifier and then led to an Einthoven string galvanometer, whose deflections were photographically recorded. In all experiments the animals were kept at $25 \pm 1.0°$ C.

RESULTS AND DISCUSSION

1. RESPONSES OF THE DARK-ADAPTED EYE

After preparation, the animal was kept in the dark for at least 1 hour, to permit complete dark adaptation. The electrical responses of the eye to light of different durations were then recorded, allowing at least 1 hour in the dark between each test exposure. To check on changes that might occur in the reactivity of the eye with time, the response to a standard 0.01-second exposure was obtained 10 minutes before each test exposure. The period of 10 minutes was sufficient to permit recovery of the eye from this check exposure of 0.01 second. Preceding each check exposure, a calibration of the apparatus was made by photographing the deflection resulting from a square wave of known potential.

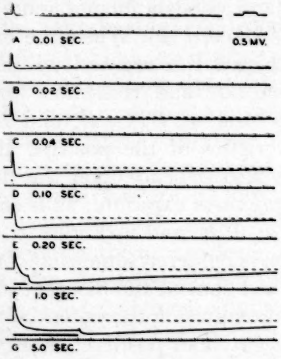

FIG. 3.—Responses of the dark-adapted eye to exposures of different durations. This series is one showing a marked positive illumination potential and a prominent positive after-potential. The magnitude and the duration of the positive after-potential depend upon the duration of the exposure of the eye to light.

The form and magnitude of the electrical response to light are dependent upon the duration of the exposure (Figs. 1, 2, and 3). For brief exposures (about 0.01–0.04 second) a negativity of the corneal lead of the illuminated eye develops rapidly (*b*-wave) and then decays. The rate of decay is exceedingly slow in some cases, leading to a persisting negative after-potential (Fig. 1, *A* and *B*). In other animals the rate of decay is rapid enough to give to the response a spike-like appearance (Fig. 3, *A*, *B*, and *C*). In the majority of the eyes, a slow positive swing is seen to follow the negative phase of the response. This positive after-potential is especially well illustrated in records *A*, *B*, and *C* of Figure 3.

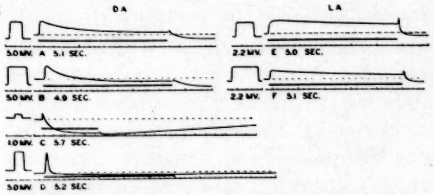

FIG. 4.—Responses to relatively long exposures of the eye to light to show the variations in response that are obtained from different animals.

When the exposure is lengthened beyond 0.04 second, a second negative phase (*c*-wave), which apparently is present in the responses to short exposures only to a minor degree, becomes increasingly prominent. With exposures of about 1.0 second or greater, the *c*-wave remains, in some cases, at a relatively high negative level, declining only slowly, as in Figure 1, *D* and *E*. In other animals the *c*-wave, with continued illumination, decays rapidly to either a low negative or a near-base-line value (Fig. 4, *A*). The decay may even proceed to such a degree that a positive illumination potential appears. This positivity of the

corneal lead is very pronounced in some eyes (Fig. 3, *G;* Fig. 4, *C*) or only slightly in evidence (Fig. 2, *E;* Fig. 4, *B*). In other words, the magnitude of the *c*-wave potential during continued illumination of the eye may be at any level from a markedly negative (about 10 mv.) to a distinctly positive (about 5 mv.) value. Accordingly, no single wave form can be selected as representing a typical wave form for *D. carolina*.

The cessation of illumination results in a number of after-potentials. One of

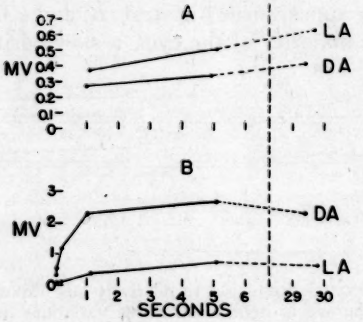

FIG. 5.—Graph *A* shows the manner in which the magnitude of the *d*-wave, in millivolts, is related to the duration of the exposure, in seconds, for the dark-adapted and the light-adapted eyes. Graph *B* shows how the magnitude of the positive after-potential is related to the length of exposure. Both these graphs were made from averaged data, graph *A* being based on the values from seven animals, while graph *B* represents the data from nine animals.

these is the *d*-wave, which has been reported for the response of both the vertebrate eye (see review by Kohlrausch, 1931) and the invertebrate eye (Jahn and Crescitelli, 1938; Bernhard, 1942). The *d*-wave in *D. carolina* is a small, rapid negative deflection (Fig. 4, *A*), absent in responses to brief exposures but increasingly prominent as the exposure is lengthened within certain limits (Fig. 5, *A*). The *d*-wave is followed by a slow, continuously changing after-potential, which may be divided, for descriptive purposes, into a negative after-potential immediately following the *d*-wave and, when it occurs, a positive after-potential occurring next in order. The relative prominence and duration of the negative and positive after-potentials vary in different animals and depend to a large degree on the duration of the previous exposure. The negative after-potential is especially well illustrated by the records of Figure 1, where it will be seen that in response to a 0.01-second exposure the negative after-potential is of small magnitude and persists for not more than 2 seconds (record *A*). When the exposure is lengthened, this negativity is increased in magnitude and continues for many seconds (Fig. 1, *B*, *C*, *D*, and *E*). The characteristics of the positive after-potentials also depend upon the duration of the previous exposure, as is shown in Figure 2, *A–E*, and in Figure 5, *B*. The situation is rendered somewhat confusing by the fact that at the moment of cessation of illumination the potential may be either negative or positive, as previously explained. It appears, however, that the positive after-potential may occur either as a positive swing from a negative level (Fig. 2, *C;* Fig. 4, *A*) or as a positive swing from a marked positive level (Fig. 3, *F* and *G*). The latter fact is extremely significant, as it tends to indicate that the positive swings during and after illumination are not merely passive processes due to an overshooting of some negative process as it decays but are, in reality, expressions of a component of positive sign.

2. RESPONSES OF THE LIGHT-ADAPTED EYE

The electrograms of the light-adapted eye of *D. carolina* are quite different from those of the same eye in the dark-adapted state. To compare the responses of an eye under the condition of dark adaptation with those of the eye in the

light-adapted state, a complete series of records, involving different durations of illumination, was first obtained with the eye dark-adapted; then a similar series was recorded after the eye had been exposed to a preliminary 20-minute adapting illumination of 290 foot-candles. The test exposure in the latter series was always made 20 seconds after cessation of the adapting illumination. Between consecutive test exposures the eye was reilluminated for a 5-minute period, the purpose being to maintain as complete a state of light adaptation as possible under the experimental condition.

The effects of light-adapting the eye of *Dissosteira* are summarized briefly. (1) There is a reduction in the magnitude of the initial negative deflection (*b*-wave) to about 5/10 to 2/10 of that obtained with the eye dark-adapted (Figs. 1 and 2; note square-wave calibrations). (2) With a long exposure the rate of decrease of the illumination potential during continued exposure is reduced, markedly with some eyes (Fig. 2, *D* and *I*, *E* and *J*) and less so in others (Fig. 1, *D* and *I*, *E* and *J*). Apparently, with light adaptation there occurs a relative increase in the magnitude of the slow negative wave (*c*-wave). This increase is so great, in the case of some eyes, that a positive illumination potential may be changed to a negative illumination potential (Fig. 2, *E* and *J;* Fig. 4, *B* and *F*). (3) The magnitude of the *d*-wave is increased, not only relative to the other phases of the electrogram, but also in absolute value, as the result of light adaptation (Fig. 1, *E* and *J;* Fig. 2, *E* and *J;* Fig. 4, *A* and *E*, *B* and *F*). This effect is seen graphically (Fig. 5, *A*) where a plot is made of the mean magnitude of the *d*-wave, in millivolts, against the duration of the exposure for both the dark-adapted and the light-adapted eye. (4) Both the negative and the positive after-potentials appear to be reduced as a result of light adaptation. This effect on the negative after-potential is seen in a very striking manner in Figure 1 when a comparison of the electrograms obtained from the light-adapted eye (*F*, *G*, *H*, *I*, and *J*) is made with the corresponding responses of the eye in the dark-adapted state (*A*, *B*, *C*, *D*, and *E*). The reduction in absolute value of the positive after-potential by light adaptation is pictured in the graphs of Figure 5, *B*.

INTERPRETATION

Two unusual features of the electrical responses obtained from *Dissosteira* merit special consideration. One of these is the marked tendency, shown by many of the eyes, for a positive deflection to occur soon after the initial negative *b*-wave. This is especially marked in the dark-adapted eye. The second feature is the relative increase in the *c*-wave following light adaptation, with the result that the illumination potential assumes a more negative value in all cases. In general, it seems justifiable to conclude that for both the illumination and the after-potentials the positive deflections are decidedly less prominent when the eye is in the light-adapted state. Information regarding the specific source or sources of the recorded potentials is inadequate, but it appears to the authors that the effects of light adaptation and the variations of the illumination potential obtained from different animals of this species are most simply explained on the assumption that two sources of potential, oppositely oriented with respect to the "active" electrode, contribute to the recorded electrogram. The wave form that is actually recorded from the illuminated eye would then depend upon relative magnitudes, latent periods, and time courses of two contributions from separate sources. Accordingly, from a

theoretical point of view, the recorded response could be the net effect of two components acting as illustrated in Figure 6. The diagrams in this figure illustrate not only how the *b*- and *d*-waves might originate but also how a difference in the relative magnitudes of two components could account for a variation in different animals of the illumination potential from a negative to a positive value. According to this type of analysis, the effects of light

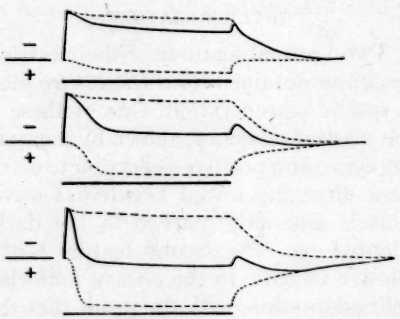

FIG. 6.—Diagrams indicating how, theoretically, two components of potential change (*dotted curves*) of opposite polarity and of different relative magnitudes and rates of change could algebraically sum up to give a resultant wave form (*solid line*) resembling the responses obtained from the eyes.

adaptation might be explained as being due to a differential action of the adapting process on two components. Without further information it is impossible to state whether light adaptation affects the response by decreasing the magnitude of a positive component or by increasing that of a negative component. The recent report of Jahn and Wulff (1942) on the grasshopper *Trimerotropis* offers some information. These workers studied the responses of the normal and deganglionated (optic ganglion) eye to light and came to the conclusion that the electrogram obtained is a summated response of two slow components—one from the optic ganglion, with positive orientation, and the other from the sensory membrane, with negative orientation. They also obtained an effect of light adaptation on the *c*-wave similar to that reported here. The effect that they obtained on the *c*-wave was largely abolished by removal of the optic ganglion. Such a result suggests that the marked effect of light adaptation in reducing the tendency for the *c*-wave to swing positive is due to an actual decrease of the positive component originating in the optic ganglion. The assumption of only two components in the makeup of the electrograms of *Dissosteira* is very probably an oversimplification. Bernhard (1942) considered the negative monophasic response from the isolated eye of *Dytiscus* as being made up of two negative components—one fast, the other slow. It may well be that the negative component indicated in Figure 6, rather than being indicated as a single element of change, might better be considered as two separate components. In fact, the responses shown in records *F*, *G*, and *H* of Figure 2, in which a distinct break in the descending limb of the response is seen, might be interpreted as indicating two negatives elements of change—one contributing a spikelike effect, and the other a more slowly changing contour. Since the introduction of two negative components would lead to details of construction not deducible from present information, the simpler analysis is preferred, with the expectation of modification as more facts become available.

SUMMARY AND CONCLUSIONS

1. With brief illumination (0.01–0.04 second), the electrical response of the dark-adapted eye of the grasshopper *D. carolina* consists of an initial negative potential (*b*-wave), which develops rapidly and then declines. In some eyes this de-

cline is slow enough to produce a negative after-potential; and in many eyes this decline continues below the base line, to give, in addition, a positive after-potential.

2. With a longer period of illumination (0.2–30.0 seconds), the response of the dark-adapted eye is characterized not only by an initial negative deflection but also by a slowly changing negative contour, or *c*-wave. With continued illumination, this *c*-wave continues to swing downward to a less negative or even to a markedly positive level, the particular level to which the *c*-wave decays varying from one animal to another. At the cessation of illumination, there occurs a small, rapid, negative deflection (*d*-wave), followed by either a long negative after-potential or, as in the response of most of the eyes, a positive after-potential. The magnitude and the duration of the negative and positive after-potentials increase as the exposure duration is increased.

3. Light adaptation affects the response in such a manner that the magnitude of the *b*-wave is decreased, the rate of decline of the illumination potential is reduced, the magnitude of the *d*-wave is increased, and the magnitude and duration of the negative and positive after-potentials are reduced.

4. An unusual feature of the electrical response of the eye of *Dissosteira* is the pronounced tendency, in most of the eyes, for a positive potential to develop shortly after the *b*-wave, especially when the eye is in the dark-adapted state. This can be exhibited during or after illumination with a long exposure or after a brief exposure. Another unusual feature is an increase in the general level of the *c*-wave or illumination potential relative to the *b*-wave as a result of light adaptation.

5. The contours of the electrograms can be theoretically interpreted on the basis that the response represents an algebraic summation of two components of potential change, one of which, as far as the orientation of its polarity is concerned, is positive; the other, negative. The variations in the electrical responses that are obtained from different animals would, on this basis, be due simply to variations in the relative magnitudes and rates of change of these two components.

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COMPARATIVE EFFECTS OF CERTAIN VITAMINS ON POPULATIONS OF GLAUCOMA PIRIFORMIS

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LITTLE is known about the relations of vitamins to the growth and metabolism of ciliates. In a few instances, specific requirements have been reported—e.g., thiamin for *Colpidium striatum* (Elliott, 1937, 1939) and *Glaucoma piriformis* (Lwoff and Lwoff, 1937, 1938); thiamin and riboflavin for *Colpidium campylum* (Hall, 1940*a*, 1942); and thiamin, nicotinic acid, and pyridoxine for *Colpoda duodenaria* (Tatum, Garnjobst, and Taylor, 1942). In addition, certain vitamins not yet identified are said to be required by *Tetrahymena geleii* (Dewey, 1941), by *Pleurotricha lanceolata* and *Stylonychia pustulata* (Lilly, 1942), and by *C. campylum* (Peterson, 1942). The effects of vitamins on the growth of populations have been considered by a few workers (Baker and W. H. Johnson, 1941; Elliott, 1939; Hall, 1940*b*, 1941; Hall and Shottenfeld, 1941; Johnson and Baker, 1943). The present observations on *G. piriformis* tend to supplement the findings of Johnson and Baker for *T. geleii*. In certain respects, the results obtained with the two ciliates are closely similar; in other respects, apparent differences probably are attributable to the use of different basal media and to differences in interpretation of data. The results obtained with *G. piriformis* show well-differentiated effects of thiamin and riboflavin on growth of populations. However, the relations of certain other vitamins—nicotinamide, pantothenic acid, pyridoxine, and ascorbic acid—to growth of *G. piriformis* are not yet clearly defined.

MATERIAL AND METHODS

The bacteria-free strain of *G. piriformis*[1] was the same as that used by Hall and Shottenfeld (1941). Growth was traced in the following media, with and without added vitamins:

GELATIN MEDIUM

Gelatin (Difco "granular")	10.0 gm.
K_2HPO_4	0.2 gm.
$MgSO_4 \cdot 7H_2O$	0.2 gm.
$CaCl_2 \cdot 2H_2O$	0.1 gm.
$FeCl_3 \cdot 6H_2O$	0.0025 gm.
Distilled water	1.0 liter

To this solution were added $MnCl_2 \cdot 4H_2O$ and $ZnCl_2$, each in a concentration of 1×10^{-7} gm. per cubic centimeter.

[1] Furgason (1940) has recently erected a new genus and species to include several ciliates of uncertain taxonomic position and has recognized several "species" as mere strains of *T. geleii*. In view of Furgason's careful work, it is possible that the ciliate referred to in the present paper as *G. piriformis* should be considered a strain of *T. geleii*. However, it may be pointed out that "*Glaucoma piriformis*," "*Colpidium campylum*," and "*Colpidium striatum*"—strains of which have been maintained in our laboratory for the last ten years—are antigenically distinct (Robertson, 1939; Tanzer, 1941) and also have shown differences in growth characteristics (Elliott, 1935*a*, 1935*b*; D. F. Johnson, 1935*a*, 1935*b*). Consequently, without implying any lack of confidence in Furgason's findings, we prefer to retain the older terminology until a satisfactory method for designation of strains has been developed. For the sake of accuracy, it should be mentioned that the strain referred to as "Hg" by Robertson (1939) was isolated by Johnson and later identified as *Glaucoma ficaria* Kahl (Johnson, 1935*a*); strain "Gp.H" is a Hetherington strain (obtained from Elliott). Cultures were sent to Dr. A. Lwoff, who transmitted the ciliates to Robertson. Through some misunderstanding, Robertson attributed isolation of these two strains to the writer.

CASEIN MEDIUM

Casein-Harris ("vitamin-free"), 1.0 per cent; the same mixture of salts as used in gelatin medium

CASEIN-PEPTONE MEDIUM

Casein-peptone (Difco Tryptone)	10.0 gm.
KH_2PO_4	1.0 gm.
Distilled water	1.0 liter

These media were tubed in 10.0 cc. amounts and then sterilized in the autoclave at 122° C. for 20 minutes.

In addition to fresh gelatin, casein, and casein-peptone media, attempts were made to prepare corresponding media with lower concentrations of vitamins. For one group of series, maintained as serial transfers only, dethiaminized casein medium was used. A 2 per cent solution of Casein-Harris was heated in alkaline solution (above pH 9.6) for an hour at 122° C. According to previous experience (Elliott, 1937, 1939; Lwoff and Lwoff, 1938; Hall, 1942), this treatment renders such a medium unsuitable for growth of ciliates unless thiamin is added. After this treatment the casein solution was diluted with an equal volume of double-strength salt solution and adjusted to pH 6.9 to produce a dethiaminized casein medium.

Another procedure involved the use of fluid from old flask cultures of *G. piriformis*. Presumably, the vitamin supply of such a fluid would be depleted to some extent. In the preparation of depleted casein-peptone medium, for example, flask cultures of *G. piriformis* were incubated for several months. The cultures were then filtered through paper (Whatman No. 12) to remove the remaining ciliates. One portion of this filtrate served as the control medium, while vitamins were added to other portions as indicated in each case. Each such medium was then sterilized in the autoclave. A comparable procedure was followed in preparing depleted gelatin medium.

All inorganic salts were "analyzed reagent" quality (Mallinckrodt). Casein-peptone (Tryptone) and gelatin were obtained from the Digestive Ferments Company; thiamin hydrochloride (Betabion), from Merck; riboflavin, ascorbic acid, calcium pantothenate, and pyridoxine, from S.M.A. Corporation; nicotinic acid amide, from General Biochemicals. The various vitamins were added, as specified, to the different media before final sterilization. Water was redistilled in Pyrex glass before use. Experimental cultures were maintained in Pyrex culture tubes (outside diameter, 16 mm.); stock cultures, in Pyrex flasks.

After sterilization, the tubes of media received 0.5 cc. inocula from a flask culture or a dilution flask of *G. piriformis*. With one exception (Fig. 3), all tubes in each group of series were inoculated from a single flask, the tubes containing different vitamin combinations being inoculated in rotation. In some instances, as indicated in the descriptions of figures, comparatively young stock cultures were used; in other cases, the stock cultures were several weeks old and presumably were beginning to suffer from vitamin deficiencies. Since inoculation of a large number of tubes usually required an hour or more, tubes fixed for determination of the initial count were so selected that each represented a proportionate lapse of inoculating time. However, significant differences were not noted between initial counts of tubes inoculated near the beginning and those inoculated at the end of the hour, and the average initial count would seem to be a reasonably accurate estimate for our experimental conditions. This might not be true for richer media supporting more rapid growth. Counts were made on two samples from each tube, or a total of twelve to sixteen samples, depending upon the number of tubes fixed. The

average count thus obtained was considered the initial count for each of the series in a given group. The initial pH of each medium was determined colorimetrically after inoculation. The remaining tubes were incubated in darkness at 24° ± 0.2° C. At each of the intervals specified in the figures, three or four tubes from each series in a group were fixed for counting, in order to trace

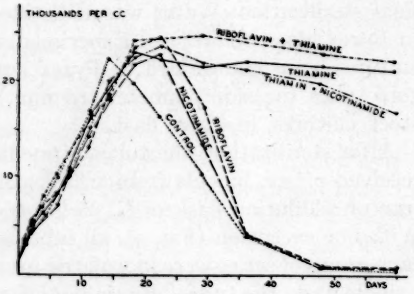

FIG. 1.—Growth of *G. piriformis* in gelatin medium; initial pH, 5.9; initial count, 1,112 ciliates per cubic centimeter. Thiamin (1×10^{-6} gm. per cubic centimeter), riboflavin (1×10^{-7}), and nicotinamide ($1\ 10^{-7}$) were added singly and in the indicated combinations. The basal medium served as the control. Inoculations were made from a 6-day stock culture in the basal medium, the stock strain having been maintained in the same medium for a number of transfers. All series were incubated concurrently. Growth is traced in point-to-point curves.

growth of the different populations. In all cases, 100 "squares" (Whipple micrometer and Sedgwick-Rafter cell) from each sample, and two samples from each tube, were counted. The significance of any apparent differences in results was determined on the basis of values of t (ratio of the difference between the two means to the standard error of the difference), as derived for small samples. In all series the counts were corrected for evaporation and thus represent density of population in terms of volume of medium immediately after inoculation.

GROWTH IN GELATIN MEDIUM

The addition of thiamin produced no significant increase in maximal density (Fig. 1). However, the later history of the population was quite different from that in the control medium, in that the density remained almost constant between 18 and 60 days of incubation. The effect of added nicotinamide was negligible except for the delay in attainment of maximal density. Riboflavin, on the other hand, produced a probably significant, although relatively small, increase in population (the value of t, 2.89, indicating less than 5 per cent probability), although the maximum was reached a few days later than in the controls.

When both thiamin and nicotinamide were added to the basal medium, maximal density was higher than in the controls ($t = 2.58$, indicating 5 per cent probability), but the increase is obviously a minor one. In contrast to the results with thiamin alone, a progressive decrease in population was observed in the second month of incubation. Upon addition of both riboflavin and thiamin, maximal density was about the same as with riboflavin alone and was significantly greater than that obtained with thiamin ($t = 4.37$, indicating 1 per cent probability). During the second month the population decreased slowly toward the level observed with thiamin alone. In both of these combinations the stabilizing effect of thiamin on maximal density is apparent. After the first month the differences between control cultures and those containing thiamin were apparent macroscopically and were very striking under a stereoscopic binocular: the cultures with added thiamin contained large numbers of active ciliates, while the controls showed relatively few.

GROWTH IN DEPLETED GELATIN MEDIUM

Upon addition to depleted gelatin medium, thiamin produced a definite increase ($t = 4.16$, indicating 1 per cent probability) in population (Fig. 2), although the maximum was much lower than that in fresh gelatin medium (Fig. 1). A comparable effect of thiamin was noted in combination with riboflavin (Fig. 2). Thiamin and calcium pantothenate, together, were apparently less effective than thiamin, although the population was still increasing at the last observation. Unfortunately, accidental destruction of some cultures prevented observations after 35 days. Upon addition of both pyridoxine and thiamin to the basal medium, growth was slower than with thiamin; but the population increased rather steadily during the last few weeks of incubation. In three of these series, maintenance of the population by thiamin was still evident after 48 days of incubation; in fact, the populations were still increasing except in the cultures containing riboflavin and thiamin.

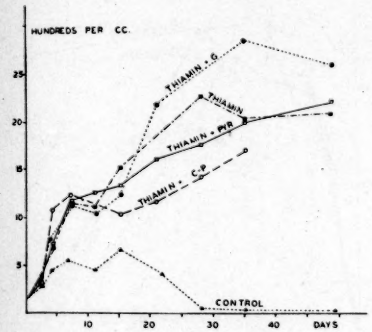

FIG. 2.—Growth of *G. piriformis* in depleted gelatin medium prepared from cultures 6 months old. The initial pH was 7.1; the initial count, 136. Inoculations were made from a 29-day stock culture in gelatin medium. Vitamins were added as indicated in the figure: thiamin (1×10^{-7} gm. per cubic centimeter); *G*, riboflavin (1×10^{-7}); *C-P*, calcium pantothenate (1×10^{-7}), *PYR*, pyridoxine (1×10^{-7}); *CONTROL*, depleted gelatin medium.

GROWTH IN CASEIN MEDIUM

The density of population (Fig. 3) was not increased significantly by the addition of thiamin to "vitamin-free casein" medium. However, the population was maintained at a fairly high level during the last 3 weeks of incubation, although a slow decrease was apparent. A significant increase ($t = 3.72$, probability less than 5 per cent) was produced by the combination of riboflavin and thiamin, the later decrease in density being somewhat more rapid than with thiamin alone.

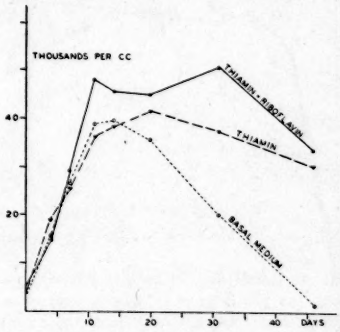

FIG. 3.—Growth of *G. piriformis* in casein medium. The series were inoculated from serial transfers in the corresponding media; hence, the initial counts, although comparable, were not identical. Initial pH was 6.9. Thiamin (1×10^{-6} gm. per cubic centimeter) and riboflavin (1×10^{-7}) were added as indicated. The series were incubated concurrently.

This effect of riboflavin on maximal density was much more apparent in dethiaminized casein medium. Growth curves were not traced for this medium, but serial transfers showed the following results. With added thiamin (1×10^{-6} gm. per cubic centimeter) the observed density ranged from 1,000 to 2,000 per cubic centimeter after different periods of incubation (12–20 days); with both thiamin and riboflavin the density has varied from approximately 20,000 to 35,000. A similar, although less marked,

effect of riboflavin has been reported (Elliott, 1939) for *C. striatum* in dethiaminized casein-peptone medium. *Glaucoma piriformis* has failed to grow in the basal medium, whereas a strain with added thiamin has been carried for 17 transfers (9 months). These results confirm previous conclusions (Lwoff and Lwoff, 1937, 1938) that thiamin is essential for growth of *G. piriformis*. In addition, the markedly beneficial effect of riboflavin and thiamin in dethiaminized casein medium indicated that both vitamins are required for production of normal population densities.

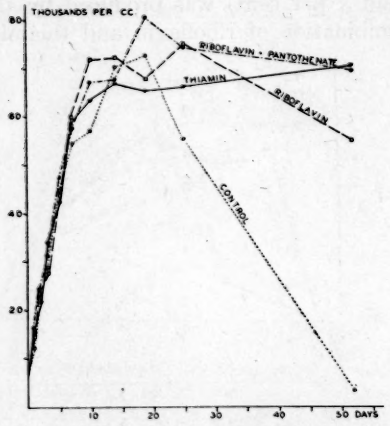

FIG. 4.—Growth of *G. piriformis* in casein-peptone medium. It should be noted that this peptone was not prepared from "vitamin-free casein." Initial pH was 6.3; the initial count, 6,406; age of stock culture in the basal medium, 43 days. Thiamin (1×10^{-6} gm. per cubic centimeter), riboflavin (1×10^{-7}), and calcium pantothenate (1×10^{-7}) were added as indicated. Casein-peptone medium served as the control.

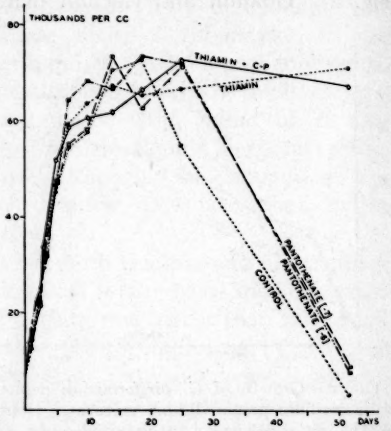

FIG. 5.—Growth of *G. piriformis* in casein-peptone medium. These series belong to the same group as those described in Fig. 4; *C-P*, calcium pantothenate (1×10^{-7}) gm. per cubic centimeter). In two other series, calcium pantothenate was added in concentrations of 1×10^{-6} and 1×10^{-7}, as indicated in the figure.

GROWTH IN CASEIN-PEPTONE MEDIUM

The addition of thiamin (Fig. 4) produced no increase in population, although a stabilizing effect on maximal density was apparent. Upon addition of riboflavin alone, there was no significant increase; however, the phase of maximal density was longer and the subsequent decline in population was much less rapid than in the basal medium. With both riboflavin and pantothenate, maximal density was reached earlier and the later decrease in density was slower than with riboflavin alone; the combination thus produced a stabilizing effect more or less comparable to that of thiamin in casein-peptone medium.

The addition of calcium pantothenate (Fig. 5) did not increase the density of population, although the phase of maximal density was somewhat longer than in the basal medium. Subsequently, the death rate was high and the populations had dropped almost to the level of the controls after 52 days of incubation. The combination of thiamin and pantothenate likewise failed to increase the population, although the stabilizing effect of thiamin on density of population was apparent.

GROWTH IN DEPLETED CASEIN-PEPTONE MEDIUM

In the basal medium (Fig. 6, control) the population reached a maximum of approximately 8,000 per cubic centimeter—a small but significant increase ($t = 3.94$, probability less than 5 per cent)—and then declined rapidly. With added riboflavin, maximal density was not significantly greater than in the control; and the later behavior of the population was comparable to that in the control medium. The combination of riboflavin and nicotinamide produced a population of about 11,600 per cubic centimeter. This represents a significant increase ($t = 3.67$, probability less than 5 per cent), as compared with the maximum in the basal medium; but the growth curve (not shown in Fig. 6) later conformed rather closely to the curves for riboflavin and the control medium.

With added thiamin the population at the maximum was more than four times that reached in the controls (Fig. 6), and at the end of 75 days (Fig. 7) the density was approximately 33,500. The combinations of thiamin and nicotinamide and of thiamin and ascorbic acid produced effects comparable to those of thiamin alone (Fig. 6). Upon addition of both riboflavin and thiamin, however, the population reached a peak (approximately 62,500 per cubic centimeter) which was approximately 75 per cent higher than that obtained with thiamin and only about 15 per cent lower than that obtained in the corresponding fresh medium (Fig. 4).

In the basal medium inoculated from a 3-day stock culture (Fig. 7) the population reached a maximum of about 22,000 per cubic centimeter on the fifth day, then dropped rather rapidly, and became stabilized at a level which was maintained for about 6 weeks. During the last month of incubation there was a

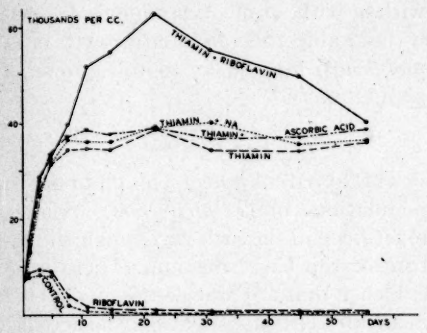

FIG. 6.—Growth of *G. piriformis* in depleted casein-peptone medium prepared from cultures 3.5 months old. Vitamins were added as indicated: thiamin (1×10^{-6} gm. per cubic centimeter); riboflavin (1×10^{-7}); *NA*, nicotinamide (1×10^{-7}); ascorbic acid (1×10^{-7}); *CONTROL*, depleted casein-peptone medium. Inoculations were made from a 21-day stock culture in fresh casein-peptone medium. The initial count was 6,427; the initial pH, 6.9. The sample of casein-peptone was the preparation used for the series described in Figs. 4 and 5.

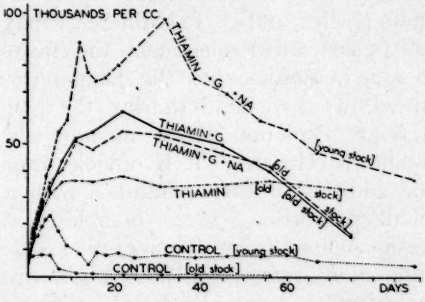

FIG. 7.—Growth of *G. piriformis* in depleted casein-peptone media. *Young stock:* cultures were inoculated from a 3-day stock culture in fresh casein-peptone medium, and the depleted medium was prepared from cultures 3 months old; *old stock:* cultures were inoculated from a 21-day stock culture, the series being homologous or identical (thiamin; thiamin and riboflavin) with those described in Fig. 6. Vitamins were added as indicated: thiamin (1×10^{-6} gm. per cubic centimeter); *NA*, nicotinamide (1×10^{-7}); *G*, riboflavin (1×10^{-7}). The initial count for the *young stock* series was 4,016; for the others, 6,427. In each case the initial pH was 6.9, and the sample of casein-peptone was the same as that used for populations described in Figs. 4–6.

gradual decrease in density, and at the end of 89 days the count was 3,517. With added thiamin, riboflavin, and nicotinamide, the density of population reached 87,455 on the twelfth day, then dropped somewhat, and later rose to about 97,240 at the end of 32 days. At 89 days the density had decreased to approximately 36,100. Maximal density thus exceeded, by approximately 30 per cent, that obtained in the corresponding fresh medium (Figs. 4 and 5). This difference may not be dependent solely upon the effects of added vitamins, since the changes produced by the ciliates during the preliminary depletion of the medium are unknown. However, it is obvious that the addition of three vitamins, with a small proportion (1:20) of relatively fresh medium from a young stock culture, more than compensated for any losses suffered by the basal medium. Since the controls reached a density of only 22,000, the increase of approximately 75,000 per cubic centimeter with thiamin, riboflavin, and nicotinamide may be attributed to the added vitamins.

In cultures started from a 21-day stock culture (Fig. 7) the effects of thiamin, riboflavin, and nicotinamide were less marked than in the preceding case. The population reached a density of about 54,500 at 22 days, as compared with an earlier maximum of approximately 8,000 in the basal medium—an increase of more than 46,000 per cubic centimeter attributable to the added vitamins. The growth curve was comparable to that for added thiamin and riboflavin (Figs. 6 and 7), and the increase in population beyond the level obtained with thiamin alone was probably produced entirely by riboflavin. At the end of 75 days the populations in the two media containing riboflavin had dropped below 20,000 per cubic centimeter, while that with thiamin alone remained about 33,500 (Fig. 7). However, considering total volumes of ciliates, the difference is not what the counts would suggest. With added riboflavin the individual ciliates were distinctly larger than those supplied only with thiamin. Such differences in size were apparent as early as the fifth day of incubation and persisted throughout the period of observation. Obviously, the combination of riboflavin and thiamin, with or without nicotinamide, is distinctly favorable to individual growth of *G. piriformis*, as well as to the growth of populations.

DISCUSSION

The apparent effects of vitamins on populations of *G. piriformis* obviously must depend, in each case, upon the nature of the basal medium. There is no evidence that any one of the media used in the present investigation was actually vitamin-free, although the depleted media apparently contained concentrations much below the optimum. With such depleted media, the results show clearly that the available concentration of thiamin is a significant factor controlling maximal density of population. Comparable results with thiamin—although less striking than in depleted media—have been reported previously for *G. piriformis* (Hall and Shottenfeld, 1941) and *T. geleii* (Johnson and Baker, 1943). In addition, Elliott's (1939) findings indicate the same significant role of thiamin in growth of *C. striatum*. In fresh gelatin, casein, and casein-peptone media, thiamin failed to increase the density of population significantly; hence, it may be assumed that the concentration of this vitamin was not the factor limiting maximal density in these basal media which have supported growth of *G. piriformis* in serial transfers.

However, one effect of thiamin is common to all the media tested by the writer. With added thiamin, the population was maintained at a comparatively high level for much longer periods than in the controls. Under certain conditions (Figs. 2, 4, and 5) populations supplied with thiamin even continued to increase, while those in the basal medium or with certain other vitamins were dying more or less rapidly. This stabilizing effect of thiamin on density of population has been observed previously (Hall and Shottenfeld, 1941) and seems to be characteristic for the media so far tested in our laboratory. It should be pointed out, however, that this particular effect of thiamin may depend, in part, upon the type of culture medium, since Johnson and Baker (1943) failed to obtain such striking results with a medium containing peptone and glucose but no added phosphate or other salts.

Riboflavin is related to growth of populations of *G. piriformis* and also to growth of individual ciliates. The available concentration of riboflavin apparently influences not only the actual density of population but also, to some extent, duration of the phase of maximal density. The relation to maximal density is obvious in the case of depleted media and dethiaminized casein medium. As might be expected, however, the addition of riboflavin to fresh culture media produced much less striking effects. The latter results are similar to the findings of Johnson and Baker—"an indication of a supplementary effect of riboflavin when added to conditioned medium with thiamin."

The moderate extension of the phase of maximal density by calcium pantothenate (Fig. 5) and the apparent stabilization produced by the combination of riboflavin and pantothenate (Fig. 4) might suggest a significant role for this vitamin. The negative results in depleted gelatin medium possibly should be discounted in view of Elliott's (1935*c*) observations that a pantothenic acid concentrate failed to accelerate growth of *C. striatum* at pH 7.0 or above. Further investigations may produce conclusive evidence concerning the relation of pantothenic acid to growth of ciliate populations.[2]

The relations of nicotinamide, ascorbic acid, and pyridoxine to growth of *G. piriformis* are uncertain, since the present data are inadequate for any definite conclusions. In view of the fact that *C. duodenaria* requires pyridoxine and nicotinic acid (Tatum, Garnjobst, and Taylor, 1942), the importance of these vitamins in metabolism of *G. piriformis* might at least be suspected. Further investigation should involve tests over a relatively wide range of concentrations, and also a consideration of possible quantitative relationships between one vitamin and another—a phase of the subject yet to be investigated for Protozoa.

Although the similarities in our results are obvious, the data for *G. piriformis* are not directly comparable to the findings of Johnson and Baker, in view of the differences in culture media. The "conditioned media" of these workers were significantly different from the "depleted media" used for *G. piriformis*, since the latter were prepared from cultures 3–6 months old, as compared with 7–15 days for the conditioned media. More striking effects of added vitamins would be expected with depleted media. Furthermore, the presence of glucose in the medium of Johnson and Baker might influ-

[2] Garnjobst, Tatum, and Taylor (Jour. Cell. and Comp. Physiol., 21:199–212, 1943) recently reported that *C. duodenaria* requires pantothenic acid for growth in pure culture.

ence growth of populations in the basal medium as well as the apparent effects of added vitamins. For instance, in experiments not described in the present paper, our casein-peptone medium with added glucose (0.25 per cent) has shown populations of more than 60,000 ciliates per cubic centimeter after 2 months of incubation. Such populations are comparable to those observed by Johnson and Baker for *T. geleii* and are in decided contrast to those noted in casein-peptone cultures of *G. piriformis* after 2 months (Fig. 4).

The marked increase in population noted by Johnson and Baker during the third month of incubation in media with certain added vitamins was not observed in our cultures. However, this apparent difference may be partly a matter of interpretation, since our growth curves are based upon counts corrected for evaporation of the medium. The volume of medium in culture tubes is progressively reduced by evaporation, sometimes by 25–30 per cent after prolonged incubation. Under such conditions a significant percentage of any apparent increase in population may be attributed to decrease in volume of the culture medium rather than to multiplication of the ciliates. Without such corrections, some of our growth curves would show later phases somewhat similar to those described by Johnson and Baker (1943). For example, in Figure 6, the uncorrected count for thiamin and riboflavin was about 66,000, as compared with the indicated 40,000 at 56 days of incubation.

The separation of "total" and "living" populations by Johnson and Baker expresses more accurately much the same results as our usual practice in counting. In our method, tube cultures are killed with a suitable fixative, and in such material the normal ciliates are distinguishable without real difficulty from those which were dead some time before fixation. Comparison of fixed and fresh material has indicated that this method is reasonably reliable, although the lag between death of the ciliates and the appearance of detectable cytological changes might tend to lengthen the apparent phase of maximal density in our cultures. In our media, "dead" ciliates begin to appear shortly after maximal density is reached, and there is a proportionate increase in the number of "ghosts" as the population grows older. However, the total of "living" and "dead" has not reached the levels observed by Johnson and Baker for *T. geleii*.

SUMMARY

So far as thiamin is concerned, the results indicate: (*a*) that this vitamin is essential to growth of *G. piriformis* and that an adequate supply is required for production of normal population densities; (*b*) that thiamin in suitable concentration exerts an important stabilizing effect upon the population, maintaining a fairly high density for relatively long periods.

Riboflavin seems to be a significant requirement for production of normal densities, bears some relation to the growth of individual ciliates, and apparently exerts at least some influence upon duration of the phase of maximal density.

Although the evidence is not conclusive, calcium pantothenate may exert an influence on the duration of the phase of maximal density and perhaps upon the actual density of population. The data for nicotinamide, pyridoxine, and ascorbic acid do not justify definite conclusions.

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NUTRITION AND GROWTH STUDIES OF AMOEBA[1]

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THIS investigation is essentially preliminary and exploratory in nature; and it must, of necessity, be so because of the scarcity of experimental evidence concerning the nutritional physiology of *Amoeba*. The logical end of such work is, of course, the establishment of the organism in a sterile synthetic medium where it can be subjected to precise physiological manipulation; but there are peculiar difficulties associated with this in the case of amoebae which are not present in the other commonly used free-living protozoans. Such an end is, nevertheless, desirable because it would furnish a controlled cell which is large and uncomplicated by

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the sexual phenomena and other specialized features of ciliates and flagellates.

It seemed advisable to approach the main problem indirectly and to attempt to clarify some fundamental factors which are operative in this form's nutrition by using methods that are somewhat crude but not too far removed from natural conditions and then correlating the facts so gained with the recently accumulated knowledge of nutritional factors in other protozoans obtained by the use of bacteria-free cultures.

In spite of the large number of experiments that have dealt with many aspects of the physiology and morphology of amoebae, a search of the literature reveals a lack of careful inquiry about the effects of dietary changes—with the one exception of Mast's (1939) work. Mast used sterile chilomonads and colpidia as food for *Amoeba proteus* and *A. dubia* and reported that "amoeba is in reference to form, size, behavior, and structure closely correlated with the kind of organisms it eats and their physiological condition." Other than this, such information as exists is fragmentary and unsatisfactorily vague. A survey of the more widely used methods for culturing amoebae (Parker, 1915; Hyman, 1917; Kepner and Whitlock, 1921; Taylor and Hayes, 1921; Edwards, 1923; Taylor, 1924; Dawson, 1928; and Halsey, 1936) shows that usually a number of possible kinds of food organisms are made available to the amoebae, that the components of this mixed diet fluctuate among themselves, and that this dietary variability between amoebae from different cultures and between amoebae taken at intervals from the same culture is often overlooked. It is to be expected from the work of Mast and Hahnert (1935) that a certain amount of selection of food organisms takes place on such a mixed diet. These investigators found that in a mixed culture of colpidia, chilomonads, and monads there is discrimination against monads only, and that, although all three are taken, the monads are indigestible.

The present investigation is concerned with the morphological and physiological effects of confining the amoebae to various diets, each of which consists of a single species of Protozoa. Since Schaeffer (1916, 1917), Dawson (1928), Hopkins and Johnson (1929), Johnson (1930), Mast (1931), Hahnert (1932), and other investigators have successfully used *Chilomonas paramecium* as a dietary staple for amoeba, this flagellate was selected to serve as a standard food organism in the present experiments. No attempt was made to work under bacteria-free conditions, partly because that would automatically eliminate possibly important factors existing in the natural environment and partly because of the practical restrictions involved in doing so. These experiments were directed more toward breaking down and analyzing, in some degree, the conditions met with *in vivo* than toward abstracting to an *in vitro* condition, in so far as this could be done, and thereby producing oversimplified and perhaps artificial results.

MATERIALS AND METHODS

CULTURING THE AMOEBAE

The original culture of *A. proteus* was purchased from the Carolina Biological Supply Company. Six specimens were removed from this culture and transferred, by means of pipettes, through 5 successive Stender dishes, each containing 15 cc. of boiled, filtered, aerated spring water. Two hundred and fifty cubic centimeters of this spring water had been boiled for 10 minutes in a Pyrex glass flask, filtered into another flask, stoppered with cotton while still

hot, and allowed to stand for 36 hours. The 6 amoebae were left in each of the above portions of spring water for 5 minutes, then transferred to another Stender dish, containing spring water, and left for 1 hour; after this each of these 6 amoebae was placed in a Petri dish that contained culture medium and food, prepared in the following manner: Twenty grains of wheat had been added to 300 cc. of tap water in a 500-cc. flask and boiled for 10 minutes; the flask was plugged with cotton while boiling and allowed to stand for 48 hours. Then the Petri dishes were filled to a depth of 6 or 7 mm.; three grains of the wheat were added, in addition to several drops of a heavy suspension of *C. paramecium* (from a wheat culture described later); and 1 amoeba was inoculated into each dish. After 3 weeks the most flourishing culture was selected for use, and subclones were cultured from this at intervals. Such cultures contained only *C. paramecium*, a fungus growing on the wheat grains, and the bacterial flora. All the specimens of *A. proteus* used throughout the experiments reported here were taken from this same stock clone.

A culture of *Chaos chaos* was obtained from the General Biological Supply House, Inc.; and a mixed culture of *A. proteus* and *A. dubia*, from the Marine Biological Station at Woods Hole. These were put, in their entirety, into Petri dishes, without adding anything to them; and specimens were taken directly from here when needed.

THE FOOD ORGANISMS

A culture of *Blepharisma* was procured from the General Biological Supply House, Inc. The other food organisms used were *C. paramecium*, *Colpidium colpoda*, *Paramecium caudatum*, and *P. bursaria;* and these were isolated from various mixed cultures in our own laboratories. The following procedure is that which was carried out for isolating, washing, and establishing in single-species culture these five species of organisms which were used as food for the amoebae. The desired protozoans were isolated by passing one to several of them at a time through the necessary number of changes of boiled, filtered, aerated spring water in Syracuse watch glasses in covered Petri dishes, both sterile. When the organism was put in one drop of spring water and found to be free of other protozoans when examined under the low power of the compound microscope, it was then put into a vaseline-sealed hanging-drop preparation in fluid from the wheat medium. These preparations were examined frequently and, after several days, if found to be satisfactory, were inoculated into several small Stender dishes containing one grain of wheat and 4.5 cc. of fluid from the wheat medium. These cultures were left undisturbed for 1 week for *C. paramecium* and *Co. colpoda*, 2 weeks for *P. caudatum* and *Blepharisma*, and a month for *P. bursaria*. These cultures were examined, and those showing a too luxuriant bacterial growth were discarded; the satisfactory ones were subcultured in larger Stender dishes that contained 20 cc. of wheat-medium fluid and two grains of wheat. It required from 2 weeks for the faster-growing to 6 weeks for the slower-growing ones to reach the density where the heaviest suspension of protozoans could be pipetted up with the least amount of culture fluid. Numerous cultures were kept going for each of the protozoans and examined frequently.

THE FEEDING EXPERIMENTS

The investigations which were undertaken fall naturally into three groups. The first group of experiments is con-

cerned with feeding *A. proteus* on exclusive diets of *C. paramecium*, *P. bursaria*, and *P. caudatum*. These experiments were undertaken simply to ascertain the suitability of these three species of food organisms as exclusive diets for *A. proteus*. Fifty amoebae were selected from the stock clone and washed by passing them through 10 baths each of 10 cc. of prepared spring water, leaving them for 5 minutes in each bath. Then 2 of these amoebae were transferred to each of 25 small, covered Stender dishes containing 2 cc. of the spring water. A drop or two of a heavy culture of the desired food organism was added to each dish; they were examined daily; and additional food was added, if needed, to maintain an abundance of food organisms. It was not necessary to standardize the number of food organisms, since Mast and Fennell (1938) found that there could be large variations in the abundance of food in the surrounding medium without appreciably affecting the frequency of ingestion by the amoebae. Three sets of 25 such preparations were set up simultaneously, using one of the species of food protozoans in each set. On the eighth day the total number of amoebae in each dish was counted. Immediately those dishes (asterisked in Table 1) with the greatest number of amoebae in each of them were selected from each of the three experiments just concluded; the washing process was repeated on the amoebae, and three sets of five preparations were arranged, with 10 amoebae to a Stender dish this time, instead of 2 amoebae to a dish as before. The 50 amoebae of each of the three nutritional strains (150 amoebae in all) were again given the diet that they had had before. These dishes were examined daily, and on the eighth day the amoebae were counted.

The second group of experiments is concerned with feeding *A. proteus* on exclusive diets of *C. paramecium*, *P. bursaria*, *P. caudatum*, *Co. colpoda*, or *Blepharisma*, the purpose being to obtain a detailed and supplementary record. The same procedure, in general, was followed in this second group of experiments as in the first. Each of these experiments was set up with 5 amoebae in each of 10 Stender dishes, but the number of amoebae in each dish was counted daily, and the experiments ran for 14 days instead of the former 8 days.

The third group consisted of offering a diet of *C. paramecium* to *A. dubia* and of confining *Chaos chaos* to exclusive diets of the chilomonas, of one or the other species of paramecia, or of the blepharisma. These experiments were, in every detail, carried out like those of the second group, and they ran at the same time; but counts were not made so frequently. This was done in order to establish some sort of comparative nutritional basis of reaction of the three kinds of amoebae.

RESULTS

FEEDING EXPERIMENTS WITH *A. proteus*

It may be seen by an inspection of Table 1 that the amoebae experienced relatively little difficulty in adjusting themselves to the conditions imposed upon them in Experiment 1, since there were only 4 apparent deaths or failures to divide. This was to be expected from the fact that they had been feeding on chilomonads in the stock culture also. In Experiment 2 the conditions met with resulted in 10 apparent failures or deaths; but here there was also a marked increase in number over Experiment 1, both in the majority of individual cases and in the total population. In Experiment 3 there were 18 apparent instances of death or division failure, and the total number was low. It is to be emphasized

that these figures on apparent fatalities and division failures are meant to give but a relative idea of the average degree of adjustment required of individu

TABLE 1

EFFECT OF EACH DIET AFTER THE FIRST 8-DAY PERIOD

Culture No.	No. of Amoebae					
	Expt. 1 Food Organism: *Ch. paramecium*		Expt. 2 Food Organism: *P. bursaria*		Expt. 3 Food Organism: *P. caudatum*	
	First Day	Eighth Day	First Day	Eighth Day	First Day	Eighth Day
1	2	5	2	25*	2	4
2	2	9*	2	34*	2	5
3	2	4	2	24	2	1
4	2	4	2	8	2	0
5	2	6*	2	12	2	6*
6	2	4	2	24	2	1
7	2	4	2	2	2	8*
8	2	6*	2	18	2	3
9	2	5	2	22	2	3
10	2	3	2	24	2	1
11	2	6*	2	6	2	8*
12	2	3	2	17	2	4
13	2	4	2	2	2	7*
14	2	6*	2	12	2	6
15	2	1	2	8	2	10*
16	2	2	2	19	2	2
17	2	4	2	13	2	6
18	2	9*	2	27*	2	5
19	2	5	2	0	2	4
20	2	7*	2	16	2	2
21	2	5	2	25*	2	10*
22	2	5	2	20	2	0
23	2	10*	2	19	2	3
24	2	7*	2	0	2	0
25	2	5	2	0	2	2
Total	50	129	50	377	50	101

*This symbol, the asterisk, marks the cultures with the largest number of individuals, and from these the amoebae were taken to be used in Expts. 4, 5, and 6. Thus, the amoebae from the nine best cultures of Expt. 1 were washed together, and the 50 needed for Expt. 4 were taken from among these; likewise, the amoebae for Expt. 5 came from the best cultures of Expt. 2, and those for Expt. 6 from Expt. 3.

amoebae by the conditions of the three experiments; they are of comparative value only, but, since they were carried out simultaneously and with exactly the same procedure, the figures should be significant as stated.

A comparison of the data summarized in Tables 1 and 2 indicates that, when a nutritional strain of *Amoeba* which had been feeding for a considerable time only on chilomonas (arbitrarily designated as the standard nutritional strain) is suddenly transferred to an exclusive diet of *P. bursaria*, then there occurs a period of adjustment to the new diet and /or the environmental conditions incidental to the administration of that

TABLE 2

EFFECT OF EACH DIET AFTER THE SECOND 8-DAY PERIOD

Culture No.	No. of Amoebae					
	Expt. 4 Food Organism: *Ch. paramecium*		Expt. 5 Food Organism: *P. bursaria*		Expt. 6 Food Organism: *P. caudatum*	
	First Day	Eighth Day	First Day	Eighth Day	First Day	Eighth Day
1	10	22	10	121	10	54
2	10	24	10	80	10	56
3	10	26	10	102	10	70
4	10	18	10	104	10	52
5	10	32	10	80	10	62
Total	50	122	50	487	50	294

diet. Also, the majority of the individual amoebae which survive this period of adjustment now reproduce, for a time at least, much more rapidly than any individual reproduced on the standard chilomonad diet. The period of adjustment required of amoebae fed on the *P. caudatum* diet was of longer duration, and fewer individuals survived it, than that required by the *P. bursaria* diet. And, furthermore, the surviving individuals from Experiment 3 finally attained (in Expt. 6) a somewhat greater reproductive rate than was standard, but still not so great as that attained by amoebae on the *P. bursaria* diet. There

does not seem to be any evidence of extrinsic toxicity in so far as the diets and their associated environmental conditions are themselves concerned, else all of the amoebae on any given diet at one time would have died, which was not the case. A certain amount of mechanical injury to the amoebae could be expected and was provided for by ascertaining that each individual was accounted for, was attached, and had fed just after the experiments were begun. What has here been called the "period of adjustment" is neither a figure of speech nor a hypothesis based only on mathematical evidence. It is a period of actual and observed morphological and physiological changes in the cell.

The experiments presented in Table 3 offer detailed corroborative evidence for the interpretations which were given to the data in Tables 1 and 2. The amoebae on the chilomonas diet exhibit no initial period of adjustment, but those on the *P. bursaria* diet clearly require such an adjustment period of at least 5 days. However, after adjustment is accomplished, the daily increase in numbers was very much greater on this paramecium than for the corresponding period on the *Chilomonas*. Also, a still longer period of adjustment was necessary on the *P. caudatum* diet—one of at least 8 days; and thereafter the daily increase was much less than on the standard chilomonad diet.

The amoebae fed on *Chilomonas* remained throughout the experiments, in all observed ways, similar both morphologically and physiologically to those of the stock culture; i.e., they fitted the descriptions in the literature given of normal *A. proteus* (Schaeffer), and these amoebae are designated "standard type." On the other hand, the amoebae on both kinds of paramecium diet are in most respects similar to each other in structure and behavior, and these are termed "*P*-type amoebae," being peculiarly different in many ways from standard amoebae. Individuals of the standard nutritional strain are typically palmate in shape; possess one contractile vacuole; are uninucleate with a biconcave discoid nucleus; and contain the usual large fat globules, differentiated refractive bodies, and truncate bipyramidal crystals. But the changes involved in adjustment to the paramecium diets resulted in *P*-type individuals which contained a very much greater proportion of the truncate bipyramidal crystals, some of which were very large; smaller and less numerous fat globules; and larger and more numerous refractive bodies, many of which were undifferentiated. Also, these *P*-type amoebae were always very much larger than a standard organism; they were much more closely attached to the substratum; and they exhibited relatively little movement. Movement was either by means of many short broad pseudopods, with no apparent main pseudopod, or by a single very broad and short fan-shaped pseudopod; and at times they exhibited the usual type of movement by a main and several secondary pseudopods. The characteristic shape of amoebae feeding on paramecia has been previously described by Mast and Root (1916*a*, *b*). In their words (1916*a*): "When Amoebae are feeding on paramecium they assume a sort of mushroom shape with a serrate edge consisting of numerous short pseudopods." Again (1916*b*):

> When the amoebae are feeding on infusoria they assume somewhat the form of a mushroom, consisting of a flat dislike portion with a serrate edge consisting of numerous short pseudopods, supported by a stalk-like basal portion. When superficially observed the animals, in this form, appear to be perfectly quiet. The pseudopods are, however, continually in a state of flux, alternately contracting and expanding slightly.

The author agrees with this description fully except for the presence of the stalk-like basal portion. This is sometimes seen; but it is not, in this investigator's experience, constant or particularly characteristic of *A. proteus* feeding on ciliates. Also, it has been quite often observed that *P*-type amoebae form so large a food cup over their prey that the entire amoeba is spread out into a thin-

TABLE 3

EFFECT OF EACH DIET AS SHOWN BY DAILY COUNTS

Culture No.	No. of Amoebae on Each of the 14 Days													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
	Expt. 7. *A. proteus* Fed on *Ch. paramecium*													
1	5	7	10	14	18	18	24	32	38	42	59	87	115	130
2	5	8	18	24	30	36	44	49	50	57	81	98	136	140
3	5	12	15	17	20	22	32	37	39	41	54	68	80	81
4	5	9	11	18	22	26	42	43	45	48	71	99	133	135
5	5	9	15	18	22	26	42	42	44	44	67	88	110	131
6	5	9	15	17	19	22	36	37	39	40	61	82	101	110
7	5	9	14	17	18	22	34	34	35	36	47	61	75	94
8	5	9	16	20	22	24	36	37	41	43	50	63	74	104
9	5	8	18	24	29	32	40	41	41	42	49	55	61	64
10	5	7	10	14	16	20	22	30	34	36	53	70	84	97
Total	50	87	142	183	216	248	352	382	406	429	592	770	969	1,086
	Expt. 8. *A. proteus* Fed on *P. bursaria*													
1	5	5	8	10	14	20	48	76	100	160	266	300	356	495
2	5	5	9	12	14	28	53	81	103	158	232	272	324	416
3	5	5	7	8	10	19	29	36	42	81	98	139	201	311
4	5	5	5	6	6	6	13	24	32	48	56	68	68	102
5	5	6	8	8	11	25	42	67	86	104	148	197	246	368
6	5	5	6	6	8	20	37	61	73	119	168	208	210	303
7	5	5	5	5	6	9	19	42	70	92	132	157	302	349
8	5	5	6	9	12	34	56	77	98	156	208	275	401	564
9	5	5	5	6	7	10	18	27	38	68	97	132	133	147
10	5	5	8	10	14	26	48	76	91	114	135	184	225	260
Total	50	51	67	80	102	197	363	567	733	1,100	1,540	1,932	2,466	3,315
	Expt. 9. *A. proteus* Fed on *P. caudatum*													
1	5	5	5	5	5	7	13	18	21	26	30	35	31	27
2	5	5	5	6	7	9	11	14	17	23	34	44	44	46
3	5	5	5	5	5	8	15	21	32	37	40	47	53	60
4	5	5	5	5	5	8	8	12	16	19	22	31	31	29
5	5	5	5	5	6	8	10	11	16	17	17	22	21	18
6	5	5	5	6	6	6	8	8	13	15	17	20	13	6
7	5	5	5	5	5	6	6	6	7	8	10	15	27	37
8	5	5	5	5	5	5	5	5	5	6	6	8	10	13
9	5	5	5	5	5	5	6	6	9	9	9	9	8	6
10	5	5	5	5	5	5	7	8	10	10	10	12	12	8
Total	50	50	50	52	54	67	89	109	146	170	195	243	250	250

walled hemisphere, but this is probably a result of a habit formed in relation to the size of the prey.

Although the amoebae fed on either kind of paramecia resemble each other far more closely than either one resembles the standard type, yet there are a few differences between these two strains of the *P*-type. Those that are confined to a diet of *P. bursaria* are uninucleate and seldom contain more than one contractile vacuole. But an estimated 10 per cent of the amoebae fed on *P. caudatum* are binucleate or, more rarely, multinucleate; and these amoebae frequently contain more than one contractile vacuole. In the former strain the zoöchlorellae associated symbiotically with the *P. bursaria* are always contained within vacuoles in the endoplasm of the amoebae which have ingested these paramecia. When an amoeba is newly subjected to this diet, the zoöchlorellae are scattered throughout the endoplasm in many small vacoules containing from one to several algae. In amoebae accustomed to the diet, however, the zoöchlorellae are confined to several large food vacuoles. In this case, the ingested paramecium is divided up among a few large food vacuoles or is digested in one vacuole; and, when digestion has been completed, this tightly packed mass of zoöchlorellae, along with a small amount of some substance in which the algae are embedded, is ejected from the amoeba. Some of the zoöchlorellae may be changed to an orange-brown or a dark-brown color while in the vacuoles, but they are always finally ejected. It is not clear whether this color change means that some of the algae have been partially digested or whether it indicates only their deaths. It appears unlikely that *A. proteus* can digest the algae, since it will starve in a medium teeming with free zoöchlorellae from which all of the paramecia have been ingested; and, also, the greater part of the ingested algae are eventually excreted unchanged. Nevertheless, there is not sufficient evidence to exclude the possibility that the zoöchlorellae may be digested to some extent. The excreted masses of zoöchlorellae are imbedded in a colorless, viscid substance which is believed to be an indigestible residue of the paramecium protoplasm. A similar occurrence takes place when the diet consists of *P. caudatum*. The cytoplasm of these amoebae contains very large spheres, which apparently, from their observed origin, consist of indigestible residues in the old food vacuoles; and these spheres also are excreted and litter the surroundings. These spheres refract light, but their relation to the refractive bodies of standard amoebae is unknown. Taylor (1924) has made a reference to these structures. She described one of her cultures of amoebae as ". . . . being adult, fine, sturdy creatures, and feeding voraciously on large Paramecia almost exclusively," and she described

> . . . . the appearance of these individuals as being extremely white in reflected light, and black looking in transmitted light. In the endoplasm were innumerable bluish spheres as large as the larger so-called excretion spheres. Nucleus-like bodies could be seen inside these spheres.

The extremely white appearance is characteristic and is due to the presence of these old food vacuoles, and the black appearance is equally characteristic because of the fact that such amoebae are stuffed with bipyramidal crystals. The "nucleus-like bodies" in the spheres were perhaps fragments of the paramecium body.

All *P*-type amoebae are characterized by the regular occurrence of a phenomenon of temporary colony formation simulating a gregaloid colony, and this

phenomenon is here called the "clumping reaction." As has been observed, this clumping reaction consists of a gathering into groups of a number of individuals; and in such a group or clump the degree of intimacy of association of the component members varies from sustained contact through fusion of hyaline ectoplasm, then of granular ectoplasm, to what seems to be full endoplasmic fusion. The largest clump ever seen was composed of more than 50 cells, but clumps composed of almost any number lower than this may occur. Some of the larger clumps have been observed to maintain themselves for at least 72 hours and probably last much longer. The cells in a given clump tend to be less intimately associated on the periphery of that clump than in the central mass; and peripheral members may wander off for various distances and may or may not return, while new arrivals may also come up and enter into the reaction. The reaction itself is very characteristic of all healthy *P*-type cultures, and it is not unusual for all of the amoebae to be taking part in one of the clumping reactions occurring in such a culture. The reaction sets in as soon as the period of adjustment has

TABLE 4

EFFECT OF *Colpidium* AND *Blepharisma* AS FOOD

Culture No.	No. of Amoebae on Each of the 14 Days													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
	Expt. 10. *A. proteus* Fed on *Co. colpoda*													
1	5	5	5	5	5	7	12	14	20	10	10	7	1	0
2	5	5	5	5	5	5	6	7	10	11	14	15	4	1
3	5	5	5	5	5	5	5	8	9	7	7	4	1	0
4	5	5	5	5	5	5	5	5	5	5	4	4	4	4
5	5	5	5	5	5	6	9	11	18	20	25	29	10	0
6	5	5	5	6	6	11	13	16	21	14	12	6	1	0
7	5	5	5	5	5	5	5	6	6	6	6	8	3	0
8	5	5	5	5	5	7	7	11	17	16	12	11	10	6
9	5	5	5	6	6	6	6	7	11	6	2	2	0	0
10	5	5	5	5	5	6	6	12	16	11	4	4	2	0
Total	50	50	50	52	52	63	74	97	133	106	96	90	36	11
	Expt. 11. *A. proteus* Fed on *Blepharisma* sp.													
1	5	5	6	6	6	6	6	6	4	4	3	3	3	2
2	5	5	4	4	4	4	4	4	4	3	3	3	4	3
3	5	5	3	3	5	5	5	5	5	5	4	4	5	3
4	5	5	4	4	4	4	4	4	3	3	3	3	3	2
5	5	5	4	4	4	4	4	4	5	5	5	5	5	4
6	5	5	5	5	4	4	3	3	3	3	3	3	2	1
7	5	5	3	2	2	2	2	2	2	2	3	3	2	2
8	5	5	5	4	4	4	4	4	4	4	4	4	2	2
9	5	5	5	5	5	5	5	3	3	3	3	3	4	4
10	5	5	5	4	3	3	3	3	3	2	2	2	2	2
Total	50	50	44	41	41	41	40	38	36	34	33	33	31	25

been completed, and it has never been observed to take place among standard amoebae. But the majority of all of these associations, of whatever degree, appear to be only temporary. Barring mechanical disturbance, a clump may persist for as long as 2 or 3 days after the food supply is exhausted. However, if no more food organisms are made available to the amoebae, the clumps will eventually largely dissociate.

Experiments 10 and 11, summarized in Table 4, were carried out at the same time as Experiments 7, 8, and 9, with the same procedure and using the same stock amoebae. Experiment 10, using *Co. colpoda* as food, shows that a long period of adjustment was necessary and that on the ninth day the highest total population of 133 amoebae was reached. So far, the figures resemble those obtained in Experiment 9, where *P. caudatum* was used as the food organism; and these amoebae fed on *Colpidium* could hardly be distinguished from those fed on *P. caudatum*, except for the fact that the ones fed on colpidia were approximately 20 per cent giant binucleate forms and contained smaller residual food vacuoles, but they possessed all of the characteristics of *P*-type amoebae. The decrease in numbers evident on the tenth day can be related to an obvious pathological condition which became plain during the last 2 days of the experiment, when the amoebae stopped feeding, rounded up while the protoplasm seemed to liquefy, and the cells burst. It is not definitely known to what this was due; but, since the colpidia exhibited no signs of any condition toxic to themselves and remained for some days after the conclusion of the experiment in a healthy condition in these same culture dishes, the amoebae may have died not from extrinsic causes but from an intrinsic toxicity of the colpidia diet.

In Experiment 11, where the *Blepharisma* was used as food, there were a very few divisions occurring sporadically throughout the period of the experiment. These amoebae conformed to the standard type except for the fact that they contained a very small percentage of platelike crystals. They seemingly experienced no difficulty in digesting the food organisms in the food vacuoles, and no obvious pathological signs were evident. This diet is believed to be either one which is barely inadequate or one requiring a very much longer period of adjustment than has been encountered before. During the 2-week period of the experiment, at least, there was evidence of neither intrinsic nor extrinsic toxicity of this blepharisma diet but a simple starvation condition.

The question now arose as to how far the effects of one of the diets producing *P*-type amoebae was due to the nutritional effects of the food organism itself and how far it was due to the medium introduced with the food organisms. Accordingly, Experiments 12 and 13, which are summarized in Table 5, were carried out as follows:

The amoebae for both of these experiments were taken from culture No. 8 of Experiment 8 on the same day that that experiment was concluded; thus these were *P*-type amoebae from the most prolific culture, that had been feeding exclusively on *P. bursaria* for 2 weeks. Immediately, 100 of these *P*-type amoebae were washed in 10 changes of prepared spring water. (Most of their zoöchlorellae were ejected during this time.) Then 50 amoebae were used to set up each of the two experiments in accordance with the usual procedure, and in Experiment 12 they were given only the standard chilomonad diet. In Experiment 13 they were also given this same standard chilomonad diet and, in

addition to this, a corresponding amount of the medium from a *P. bursaria* culture. This added medium contained free zoöchlorellae, which are always abundant in such media, but no paramecia. A comparison of the results of these two differences were very small. As a whole, the differences between the results of the two are not great enough to conclude that either experiment offered more favorable conditions than the other. Therefore, it was not the medium or the

TABLE 5

EFFECT OF FREE ZOÖCHLORELLAE AND CULTURE MEDIUM

Culture No.	No. of Amoebae on Each of the 14 Days													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
	Expt. 12. *P*-type *A. proteus* Fed on *C. paramecium*													
1	5	11	18	47	80	132	154	199	258	292	318	357	386	441
2	5	11	20	43	66	115	188	254	331	354	387	420	444	482
3	5	9	18	36	62	139	184	261	313	318	320	326	330	332
4	5	14	23	51	97	131	197	271	391	429	461	514	573	632
5	5	9	16	34	63	89	137	200	291	311	343	359	382	430
6	5	12	18	69	84	116	159	217	274	308	345	374	410	453
7	5	9	17	62	73	98	171	228	297	315	348	395	431	493
8	5	7	12	29	49	106	155	181	202	234	248	267	276	309
9	5	10	16	33	60	112	145	190	267	287	344	397	456	501
10	5	6	14	34	69	96	166	195	248	306	377	461	535	615
Total	50	98	172	438	703	1,134	1,656	2,196	2,872	3,154	3,491	3,870	4,223	4,688
	Expt. 13. *P*-Type *A. proteus* Fed on *C. paramecium* Plus an Equal Amount of Medium from a *P. bursaria* Culture													
1	5	6	10	23	38	53	88	114	135	193	245	294	368	456
2	5	9	20	37	55	94	135	167	199	233	281	353	401	470
3	5	8	18	39	62	84	102	156	190	239	304	384	466	536
4	5	10	20	56	73	89	155	238	322	397	461	511	589	682
5	5	9	19	33	54	73	97	144	171	246	298	371	443	541
6	5	9	17	29	55	102	158	193	247	289	325	368	414	470
7	5	7	18	39	66	102	164	194	220	257	293	320	362	402
8	5	11	28	48	79	92	160	188	239	241	294	332	375	427
9	5	9	16	41	54	90	147	190	231	258	287	300	316	323
10	5	6	24	62	85	115	174	255	328	368	393	411	438	473
Total	50	84	190	427	621	894	1,380	1,839	2,282	2,721	3,181	3,644	4,172	4,780

experiments shows that the corresponding daily increases in total number were somewhat higher in Experiment 12 (chilomonad diet only) than in Experiment 13 (chilomonad diet plus paramecium culture medium containing zoöchlorellae), with the exceptions of the third and fourteenth days, when the zoöchlorellae free in it that was responsible for the effects produced by the *P. bursaria* diet; but these effects must be due to the intrinsic nutritional value of the digested paramecia in symbiotic association with the zoöchlorellae. In neither experiment was there any period of adjustment evident; and the amoebae

reverted, in so far as they could be observed in culture, to the standard type at the beginning of the experiments. But in both experiments the increase in numbers was considerably higher than it was when standard (stock) amoebae were first introduced to the *P. bursaria* diet (Expt. 8, Table 3), and the increase was very much greater than when standard amoebae were confined to the standard chilomonad diet (Expt. 7, Table 3).

TABLE 6

RATE OF INCREASE OF STANDARD AND *P*-TYPE AMOEBAE

Day	Percentage of Increase			
	Expt. 7 Standard *A. proteus* Fed on *Chilomonas*	Expt. 8 Standard *A. proteus* Fed on *P. bursaria*	Expt. 12 *P*-type *A. proteus* Fed on *Chilomonas*	Expt. 13 *P*-type *A. proteus* Fed on *Chilomonas* Plus Medium from Culture of *P. bursaria*
1.....	74.0	2.0	96.0	68.0
2.....	63.2	31.4	75.5	126.2
3.....	28.9	19.4	154.6	124.7
4.....	18.0	27.5	60.5	45.4
5.....	14.8	93.1	61.3	44.0
6.....	41.1	84.3	46.0	54.4
7.....	8.5	56.2	32.6	33.3
8.....	6.3	29.3	30.8	24.1
9.....	5.7	50.6	9.8	19.2
10.....	37.9	40.0	10.7	16.9
11.....	30.1	25.5	10.8	14.6
12.....	25.8	27.6	9.1	14.5
13.....	12.1	34.4	10.8	14.6

Table 6 gives the daily percentages of increase for Experiments 7, 8, 12, and 13. From these figures can be seen the unusually high percentage of increase during the first 3 days of Experiments 12 and 13; then a gradual fall thereafter. It is possible that during the later days of these two experiments the populations were overcrowded and that this, in part, depressed division. These amoebae were voracious and required very frequent additions of chilomonad suspensions to their culture dishes; and accumulated metabolites and CO_2 may have had an adverse effect, for it was found, upon continuation of these cultures beyond the fourteenth day, that the amoebae died out within 60 hours unless they were washed and transferred to fresh cultures. If this were done, they continued to divide vigorously. The slightly higher percentage of increase during the last 5 days of Experiment 13 over that for the same period in Experiment 12 is thought to have been due to the presence of the zoöchlorellae in the former experiment, bringing about a more favorable O_2/CO_2 equilibrium there.

Nutritional strains of these amoebae feeding exclusively on chilomonads have been continued for as long as 7 months with the usual procedure. Strains feeding on *P. bursaria* have likewise been continued for as long as 3 months. So it seems probable that either food organism in this type of culture furnishes a complete diet for *A. proteus* upon which the amoebae could multiply indefinitely. But efforts to continue strains confined to a diet of *P. caudatum* have never been successful for more than 1 month at most, the amoebae dying out after passing through a pathological phase similar to that observed in Experiment 10, where colpidia served as the food. Long-term cultures have not been attempted with any other single kind of food organism. Repeated attempts have been made to culture *A. proteus* on an exclusive diet of *Euglena gracilis* or *E. anabaena*, but these foods have been found to be largely or entirely indigestible—certainly they will not support growth of *A. proteus* as exclusive diets.

FEEDING EXPERIMENTS WITH *A. dubia*

The stock culture of *A. dubia* was not a clone culture of this form, and it also contained *A. proteus*. At first the food

consisted almost entirely of a mixture of several Phytomastigida, such as *Astasia*, *Euglena*, and *Chilomonas*, with the colored forms predominating—all in hay-infusion medium. Thirty-nine specimens of *A. dubia* were selected from this culture, washed, and transferred to a small Petri dish containing prepared spring water, to which a suspension of the usual chilomonads (raised in boiled wheat medium) was added. After 10 days, these had increased to only 55 amoebae. Fifty of these were washed, and 5 were placed in each of the usual ten experimental cultures and fed on chilomonads. The daily counts showed a steady decrease in numbers down to a total of only 22 individuals on the seventh day, and on the twelfth day all of the amoebae had disappeared. Meanwhile, several grains of wheat had been added to the stock culture. The fauna in the stock culture had, at the same time, become predominantly ciliates and rotifers; and the *A. dubia* died out, while the *A. proteus* continued to multiply. The only changes observed in the *A. dubia* confined to a chilomonad diet were an increase in the amount of bipyramidal crystals over the other types of crystals and the loss of their characteristic "feathery" appearance, caused by a reduction in the number of their pseudopods, which became shorter, broader, and heavier.

FEEDING EXPERIMENTS WITH *Ch. chaos*

The stock culture of *Ch. chaos* was also not a clone culture. It consisted of a hay-infusion medium, and the food organisms were principally *P. caudatum*, *Frontonia*, *Colpidium*, and other ciliates. When this culture first arrived through the mails, it contained no large forms at all; but, upon standing for a week, as directed by the distributors, the large *Chaos* forms made their appearance. Two hundred amoebae were taken from this stock culture, washed, and used to set up Experiments 14–17 according to the usual procedure. Only the largest individuals were selected for use in the experiments, and only the total daily counts are given in Table 7.

TABLE 7

THE MULTIPLICATION OF *Chaos* ON DIFFERENT DIETS

Food Organism	Total No. of *C. chaos* Present on Each of 14 Days											
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 11	Day 12	Day 13	Day 14	Day 33
Expt. 14, *C. paramecium*	50	52	69	85	85	90	90	100	105	108	109	51
Expt. 15, *P. caudatum*	50	61	90	116	145	184	231	322	404	488	652	?
Expt. 16, *P. bursaria*	50	58	59	59	63	64	?	?	56	55	57	0
Expt. 17, *Blepharisma* sp.	50	50	51	55	58	58	?	?	?	43	39	0

The *Ch. chaos* cultured on chilomonads became gradually smaller, until on the fourteenth day they were, with the exception of one individual, no larger than *A. proteus*. Also, 30 smaller-than-average specimens of *Ch. chaos* were taken from the stock culture and confined to a chilomonad diet; at the end of a 3-week period they had more than doubled in number, but all of them had decreased in size until these, too, were no larger than *A. proteus*. Always, on this chilomonad diet, the *Ch. chaos* assumed the shape of standard *A. proteus* with clavate pseudopodia; no evidence of clumping could be seen; and there was a high degree of motility. The amoebae

feeding on *P. caudatum* exhibited the characteristic *P*-type mushroom shape, decreased motility, and pronounced and constant clumping reactions; "individual" organisms were enormous. Full protoplasmic fusion was especially common in this form. Thus, the chilomonad diet imposed on *Ch. chaos* produced again what has been termed "standard amoebae," and the *P. caudatum* diet gave rise to *P*-type amoebae—at least as far as external appearance and behavior are concerned. No comparative study was made of the cytoplasmic inclusions. On a diet of *P. bursaria* the behavior and shape of the amoebae was that of the *P*-type; but the organisms were below average in size, though not so small as those feeding on chilomonads which eventually decreased to the size of *A. proteus*. Furthermore, these amoebae finally ceased even to ingest the *P. bursaria*, and they then died out. On the *Blepharisma* diet the amoebae appeared to be slowly starving to death, though at first they did ingest the food organisms and appeared to digest them in the food vacuoles; but they apparently were only nutritionally deficient as a diet, not toxic in any way.

DISCUSSION

It must be emphasized that these experiments have been carried out as nearly as possible under natural conditions, with all of the consequent disadvantages of dealing with a number of uncontrolled variables, but also with the advantage of isolating, clarifying, and defining the problems connected with the nutrition of these amoebae with the certainty that synthetic results have not been created even before the problems occurring *in natura* are recognized.

Mast (1939), using sterile chilomonads as the sole source of food for *A. proteus*, found that they would not support continued multiplication of the amoeba, although chilomonads raised in glucose-peptone solution were more nearly adequate as food than those raised in ammonium acetate solution. He found that an exclusive diet of the sterile chilomonads caused a great increase in size and number of differentiated refractive bodies; that the bipyramidal crystals also increased greatly in number, while the quantity of fat decreased; and that these amoebae were "normal in size, form, and activity." The present investigation, however, has shown that chilomonads raised in a wheat medium along with bacteria offer an entirely adequate diet upon which this amoeba can multiply probably indefinitely, while the amoebae remain normal in every respect. Here wheat as the source of organic nutriment may have produced chilomonads chemically so constituted that they were entirely adequate to fulfil all the nutritional requirements of the amoeba, and this may explain the matter fully. However, although chilomonads are not bacteria-feeders, it is possible that bacterial action on the medium affected the chemical constitution of the chilomonads or that such bacterial metabolites affected the amoeba directly. The effect of bacteria as a direct food for *A. proteus* has not been taken into account, since it has been found that the amoebae invariably starve after they have exhausted the protozoan prey from the medium. Halsey (1936) tested bacterial media as to their suitability for both *A. proteus* and *A. dubia* and showed that the amoebae died out in such media unless protozoan food organisms were also made available. Likewise, Dawson (1928) considered that the main thing to guard against in culturing amoebae was a too luxuriant bacterial flora. Miller (1937) stated that "certain bacterial forms of the culture medium are capable of invading the

cytoplasm of Amoeba without showing any signs of deformation or effects of digestive processes." Furthermore, bacteria can usually be observed in small numbers in the cytoplasm of any specimen of *A. proteus*, alive and unharmed. These bacteria may be there only by accident, and there is no evidence that they are customarily digested. Also, the amoebae used by Mast (1939) probably contained bacteria in their cytoplasm throughout his experiments, though he makes no statement about this. In addition to the question of bacteria, there yet remains the possibility that in the author's work the small amount of wheat-medium fluid carried over when chilomonad suspensions were added as food to the experimental cultures may have been sufficient to provide the amoebae directly with dissolved substances necessary to their growth. In the light of the preceding statements, therefore, the view is taken that the wheat grains probably supply, either directly or via the chilomonad protoplasm, the chemical substances necessary for continued growth of *A. proteus*. The present experiments do not give any precise chemical knowledge of the growth requirements of *A. proteus*, but they do indicate that the required substances seem to be present in wheat grains but absent from glucose-peptone solutions and that chilomonads do not synthesize them when raised in such solutions. The role of bacteria is still very obscure, and it may not be negligible; it is considered so here simply for convenience of discussion.

As to the adequacy of chilomonads as an exclusive diet for *A. dubia*, the results show that even chilomonads raised in nonsterile wheat medium do not support continued multiplication of this species. Mast (1939) stated that sterile chilomonads raised in either ammonium acetate or glucose-peptone solution were inadequate as food for this amoeba for any length of time. He also stated that his specimens fed on these sterile chilomonads showed a great increase in the number of irregular (roughly bipyramidal) crystals, and this has also been shown to occur on the nonsterile chilomonad diet. Further, the present experiments indicate that there exists in green flagellates a substance or substances necessary for the growth of *A. dubia*. Schaeffer (1916) reported that "notwithstanding the wide range of diet, small flagellates such as chilomonads appear to form the larger part of the food of granular amebas; while diatoms and desmids constitute the greater part of the food of raptorial amebas." He was referring to what he then called "granular amebae" and would now call *A. proteus*, since raptorial amoebae are now called *A. dubia* (Schaeffer, 1917).

The nutritionally induced modifications in the morphology and physiology of *A. proteus* which are the direct outcome of confining the amoebae to exclusive diets of either colpidia or one or the other species of paramecia present essentially the same picture no matter which of the three foods is used. This basic similarity between the effects produced by these three diets consists of a great increase in the amount of truncate bipyramidal crystals; an increase in the amount of refractive bodies, with an accompanying tendency for a large proportion of these bodies to be undifferentiated; an increase in the size of individual (uninucleate) cells; and decreased motility in conjunction with the clumping reaction. Mast and Doyle (1936*a*, *b*) have presented much experimental evidence concerning the cytoplasmic inclusions of *A. proteus;* they are of the opinion that the bipyramidal crystals are probably a magnesium salt of a substituted glycine. They also

found that the differentiated refractive bodies consist of an outer layer which is a protein stroma impregnated with lipid containing fatty acid, that the shell is composed of a substance which is not starch or glycogen but has carbohydrate properties, and that the nature of the central fluid is not known. When Mast (1939) placed *A. proteus* upon an exclusive diet of *Co. striatum* raised in sterile tryptone-phosphate solution, he found that the amoebae became very large with many fat globules and that many had two nuclei. Also, Mast stated that some of his cultures of colpidia contained a mold upon which the colpidia fed. He believed that *A. proteus* could multiply indefinitely upon such a diet of colpidia. It is obvious here that there are distinct differences in the nutritional effect on *A. proteus* of colpidia (*Co. striatum*) raised in the absence of bacteria, as in Mast's experiments, and colpidia (*Co. colpoda*) feeding directly on a mixed culture of bacteria, as in the present experiments. The presence of mold in Mast's colpidia cultures is also a complicating factor. On both kinds of colpidia there was an increase in the size of the amoeba, decreased motility, a tendency to form undifferentiated refractive bodies, unusual types of pseudopods and locomotor activities, and a tendency toward more than average binucleation. At the present time the differences between the effects produced on the amoeba by the two colpidia cannot be explained, but they are probably due both to the fact that there are specific differences between the two colpidia and to differences between colpidia raised on bacteria and others raised in the absence of bacteria.

It is significant that, when Mast fed *A. proteus* on sterile chilomonads, he found this diet inadequate for continued multiplication of the amoebae; and there was a great increase in bipyramidal crystals and a great increase in size and number of sharply differentiated refractive bodies, and the fat decreased. Then, in the present work, when *A. proteus* was fed on nonsterile colpidia, the crystals again increased greatly; the refractive bodies increased and many of them were undifferentiated, while the fat decreased, and the diet appeared to be intrinsically toxic. So, with a diet of *P. caudatum* the colpidia effects were repeated; and the diet appeared to be toxic for intrinsic reasons, after a slightly longer period. It is interesting to speculate upon the possibility that the glycine in these bipyramidal crystals serves one of the functions that glycine serves in mammalian physiology, i.e., it detoxicates acids by conjugating with them. (According to this idea, the same thing may take place in *A. dubia*, where both Mast and the present investigator have shown that both sterile and nonsterile chilomonads are inadequate as an exclusive diet and bring about an increase in the bipyramidal crystals here also.) On the other hand, according to Mast, *A. proteus* can live indefinitely on sterile (mold present) colpidia, which cause the bipyramidal crystals to decrease greatly, the spherical bodies to become scarce and undifferentiated, and the fat to increase. Thus, an increase in bipyramidal crystals can be correlated directly with diets which are inadequate or intrinsically toxic, while a decrease in the crystals may simply mean that less than the standard amount of detoxication is required by Mast's colpidia diet. A decrease in amount of the fat may be directly correlated with inadequacy or toxicity of the diets and also with increase in amount of crystals. An increase in refractive bodies is less plainly correlated with decrease of fat, increase of crystals, and dietary inadequacy or

toxicity. Differentiation of the refractive bodies, or undifferentiation, cannot be correlated with anything else. As would be expected from the complicated nature of these bodies, which was demonstrated by Mast and Doyle (1936*a*), the qualitative changes are probably more important here than the quantitative, and their correlation with other cytoplasmic elements must await a better understanding of them.

While the three *P*-type diets have been shown to produce many of the same effects, the *P. bursaria* diet differs from the other two by being nontoxic and apparently fully adequate for an indefinite length of time and by causing no polynucleation and no increase in the number of contractile vacuoles. This difference is possibly related to the fact that *P. bursaria* is easily grown in sterile media (Loefer, 1934), while *P. caudatum* has not yet been raised in the absence of other organisms; but even *P. bursaria* will not grow in sterile media when deprived of its symbiotic green algae. There seems to be a factor, or group of factors, in the association of this paramecium with its zoöchlorellae, which allows it to grow in sterile liquid media in the absence of bacteria; and this unknown factor or group of factors presumably counteracts the toxicity of the *P*-type diet for *A. proteus*. It is not clear whether this effect is transmitted to the amoebae through the digestion of the paramecium protoplasm, through the digestion of the zoöchlorellae themselves, or a more indirect effect due to the presence of the algae in the amoebae's cytoplasm.

The *P. bursaria* diet has been shown to be associated with a much higher division rate than is standard. A *P*-type cell is considered to be one in which the standard nutritional balance has been upset, producing a physiologically and morphologically abnormal cell; and only when the diet consists of *P. bursaria* can this abnormal cell accomplish the necessary adjustment of its physiological mechanisms in order that it may grow and multiply indefinitely at its new physiological level. A diet of either *P. caudatum* or *Co. colpoda* apparently brings about the same *P*-type disequilibration, resulting in an intrinsically toxic condition associated with polynucleation and an increase in number of contractile vacuoles; but these diets are deficient in the factors necessary for adjustment to a new physiological level. It is interesting here to recall that Chalkley and Daniel (1934) reported that lactic and pyruvic acids both acted as inhibitors of cytoplasmic division in *A. proteus* but did not affect nuclear division; and they produced binucleate amoebae in this way that continued to live and later divided again. So it is not difficult to conceive of some such effect as this being operative in the abnormal metabolism nutritionally produced in the present experiments. But, once this new level has been attained, it seems to result—for a time at least—in a much higher division rate than is normal. However, none of the experiments are of sufficient duration to draw any final conclusions as yet about the length, permanency, or retention after a return to standard conditions of this increased reproductive rate. The results are suggestive, but additional experimentation is necessary. It may be said that the explanation offered here is intended to be only a very tentative one, to serve merely as a working hypothesis.

The clumping reaction has not previously been reported for *A. proteus*. However, the same phenomenon has been observed in this laboratory by Reynolds (1938–39) in *Arcella vulgaris;* by Looper (1928) in *Actinophrys sol*, and

also by Kepner (1925) in *Ac. sol.* Elsewhere, Sondheim (1916) described a similar phenomenon in *Ac. oculata.* These last three authors have ascribed the phenomenon to the necessity of capturing large objects of prey; i.e., they considered it to be simply a temporary morphological adaptation. It is revealing that in Looper's work his experimental organisms were cultured in wheat medium containing chilomonas, colpidia, paramecia, and various hypotrichous ciliates. And he found that temporary colonies would form by fusion of individuals, "provided the food supply consists chiefly of rotifers or large protozoa." He also stated that this fusion involved only the ectoplasm and not the endoplasm. The present investigator is himself in doubt as to the significance of this clumping reaction, but it certainly cannot be dismissed as simply a temporary morphological adaptation necessitated by the size of the prey. The reaction has been shown to be but one of a closely linked group of nutritionally induced morphologic and physiologic changes which *in toto* constitute a radical departure from the normal condition of the *A. proteus* cell.

That part of the work wherein *Ch. chaos* was used as the experimental organism suggests that we are here dealing with a form very closely related to *A. proteus. Chaos chaos* can perhaps best be looked upon as a variant from the standard *A. proteus* type, in which the original physiological disequilibration brought about by reason of the *P*-type diet has been readjusted by means other than those employed by *A. proteus* feeding on *P. bursaria.* It is not clear whether the polynucleation in *A. proteus* which was produced by a diet of either *P. caudatum* or *Co. colpoda* was due to nondisjunctive fission or to a more permanent type of clumping reaction. But the evidence now at hand indicates that the *chaos* form is one in which the clumping reaction is tremendously accentuated. The information now available is not sufficiently extensive to do more than suggest the directions which further research may profitably take.

The problems uncovered by these preliminary investigations into the nutrition and growth of the free-living amoebae indicate that results of general importance may be obtained from continued work along these lines. One example of this is the interesting possibility of there being a fundamental relationship between the phenomena exemplified by *Ch. chaos* and the clumping reaction, the foreign-body giant cells of the reticulo-endothelial system of mammals, and phagocytosis in general. Amoeba may prove to be valuable intermediary material by means of which recent advances in the knowledge of the nutritional physiology of flagellates and ciliates may be integrated with the physiology of higher forms.

SUMMARY

1. Specimens from a clone culture of *A. proteus*, raised in a wheat medium containing *C. paramecium*, were confined to exclusive diets of several different protozoan food organisms that had also been raised in a wheat medium.

2. On an exclusive diet of *C. paramecium* the amoebae remained normal and continued to grow and multiply indefinitely; and this diet was found to be entirely adequate.

3. On a diet of either *P. bursaria*, *P. caudatum*, or *Co. colpoda* the amoebae exhibited a great increase in the amount of truncate bipyramidal crystals, an increase in the amount of refractive bodies with a tendency toward undiffer-

entiation of these bodies, an increase in size of the whole cell, a decreased motility, and a characteristic clumping of cells into plasmodial masses.

4. An exclusive diet of *P. bursaria* seemed to be fully adequate for continued growth and multiplication of the amoebae, for an indefinite length of time. But a diet of either *P. caudatum* or colpidia was apparently intrinsically toxic and was associated with polynucleation and increase in the number of contractile vacuoles. The amoebae grew and multiplied to a slight extent on these two toxic diets but eventually died.

5. The *P. bursaria* diet brought about an increased reproductive rate—at least during the two-week period of the experiments; but the results were inconclusive when such amoebae were returned to a chilomonad diet, although they indicate that this increased rate is retained for a time.

6. A diet of *Blepharisma* was probably inadequate for continued multiplication of amoebae, but it was not toxic.

7. Specimens from a nonclone culture of *A. dubia* in hay-infusion medium were fed on an exclusive diet of chilomonads, but the amoebae multiplied for a short time only and then died out entirely. The experiments with this amoeba suggest that green flagellates supply a necessary part of their diet.

8. Specimens from a nonclone culture of *Ch. chaos* were fed on exclusive diets of several protozoans. A chilomonad diet caused a great decrease in the size of the *chaos* organism. A diet of *P. caudatum* brought about a great increase in the size of the organism and in its rate of division, and this diet was accompanied by typical clumping reactions. The *P. bursaria* diet produced amoebae which clumped, but the individual organisms were not so large as those feeding on *P. caudatum*. The *Blepharisma* diet was inadequate.

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EFFECT OF INTERRENALECTOMY ON SOME BLOOD CONSTITUENTS IN THE SKATE[1]

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Most of our knowledge of the effects of adrenal ablation has come from removal of the whole gland or of the medulla. Removal of the cortex without destruction of the medulla is impossible in the mammal. In the elasmobranchs, interrenal and chromaffin tissues (the homologues of mammalian cortex and medulla) are separate. In certain species the interrenal tissue is sufficiently concentrated so that it can be completely removed, leaving the chromaffin tissue intact. Interrenalectomy in the torpedo and in the skate has been performed by Biedl (1913) and in the torpedo by Kisch (1928) and by Dittus (1937). The resulting symptoms were described, but no other observations were made.

In most mammals adrenalectomy is followed by a decrease in plasma sodium and a rise in plasma potassium. The blood urea rises, and the blood sugar falls. These changes do not occur when the medullary tissue alone is destroyed. Recently we have made a study of the plasma sodium and potassium, blood urea, and sugar and a few observations on the liver glycogen in the skate after interrenalectomy. Changes due to inanition, as well as to the operation itself, have been considered.

METHODS

We have interrenalectomized *Raja laevis*, *R. eglanteria*, *R. diaphanes*, and *R. erinacea*. The first species proved im-

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practicable because interrenalectomy injured too much of the kidney. *Raja eglanteria* is the easiest to operate upon, since the interrenal tissue is the most concentrated of all species examined, except possibly certain species of *Torpedo*. However, *R. eglanteria* withstands operation poorly. We did not use many individuals of *R. diaphanes*, because they were not available. Individuals of the species *R. erinacea*, being the most plentiful and the most rugged, were employed for this work except where indicated.

The animals were collected from fish traps or by dredging. Occasionally they were caught with a hook and line. Some were kept in shallow wooden tanks at the Bureau of Fisheries Laboratory, while others were kept in a special large lead-lined tank in water maintained at 10°–12° C. at the Marine Biological Laboratory. Large tags of white celluloid, marked with black lacquer, were tied by string through the pectoral fin, so that the numbers could be read easily at a distance. The string was inserted by means of a surgical needle and tied on the side opposite the tag. The animals would not eat voluntarily and were not forcibly fed. Blood was drawn from the central caudal vein, clotting being prevented by heparin.

Glucose-tolerance curves were determined on one interrenalectomized and two control skates. For these experiments glucose (1.5 cc. per kilo of a 10 per cent solution) was injected into a lateral tail vein, the blood samples being drawn from the central caudal vein, as usual. Blood-sugar determinations were made by the Shaffer-Hartman method as modified by Somogyi (1937).

Glycogen determinations were made in a few normal liver biopsies. For this purpose 2–4 gm. of one lobe was sliced off and the bleeding stopped with "cold" cautery. Otherwise, glycogen determinations were made only at the time the animal was sacrificed. The whole liver was rapidly excised, dropped into 30 per cent KOH, and digested. Aliquots of this digest were taken for determination of glycogen by the method of Good, Cramer, and Somogyi (1933).

For operating, the animal was fastened, ventral side up, to a concave board by clamping thin boards on the pectoral fins. Where the animal came in contact with the boards, several thicknesses of newspaper, soaked in sea water, were used as pads. Running water reached the gills through a tube inserted into one spiracle. The board was tilted so that the water spilled into an outflow tray and thence to a wastepipe. Operations were performed as nearly aseptically as possible. The skin was swabbed with 70 per cent alcohol. Sterile cloths covered the animal, and the instruments were sterilized with alcohol. Approach was through the mid-line of the abdomen. The interrenal tissue was destroyed by "cold" cautery after dissecting as free as possible. Sometimes the interrenal tissue could be picked out after dissection without resort to cautery. Muscle and skin were stitched separately with simple discontinuous sutures. Control animals were operated in a similar fashion except that the interrenals were not removed. The same length of time was employed as when interrenalectomy was performed.

Interrenalectomized and sham-operated animals were kept as long as the stitches remained intact, but, as soon as there was evidence of their beginning to give way, samples of blood were taken. This occurred at 1 week or later. In some instances the animals were sacrificed before the stitches made it necessary. Thus it was impossible to determine the survival time which they would have attained had they been left undisturbed.

It would have been a few days longer at most.

The blood picture was studied by comparing, in each animal, the blood sample taken just prior to interrenalectomy with a second sample drawn at some time during the postoperative period. The second blood sample is italicized in Tables 1, 2, and 3. Since it was found that sex did not alter the blood picture, no distinction was made on this basis.

The animals were divided into three groups, as follows: (1) the sham-operated or control animals; (2) the interrenalectomized animals that were quite active at the time of drawing the second blood sample; and (3) interrenalectomized animals that were in the late stage of insufficiency, i.e., very sluggish, at the second sampling.

RESULTS

Sham-operated (control) skates (Table 1).—Ten skates were studied after sham operations. In two (Nos. 65 and 75) the second blood sample was drawn when the animal was moribund. The others were in good condition at the time of taking the second blood sample.

Plasma sodium rose in all but three of the controls. One (No. 77) showed little change; two (Nos. 63 and 72) gave per-

TABLE 1*

BLOOD CHANGES IN SHAM-OPERATED (CONTROL) SKATES

No.	Days after Operation	Sodium (mEq/l)	Potassium (mEq/l)	Calcium (mEq/l)	Magnesium (mEq/l)	Chloride (mEq/l)	Urea (mM/l)	Erythrocytes (Volume per Cent)	Glucose (Milligrams per Cent)
63	2.5	265	8.1	12.3	2.0	258	342	21	57
		251	.....	*11.6*	*6.2*	*250*	*312*	*19*	*53*
72	8	285	4.8	11.6	.....	250	304	17	69
		244	*6.6*	*12.0*	*4.7*	*262*	*312*	*15*	*16*
73	8	244	.....	13.4	5.2	253	335	20	67
		264	*5.6*	*13.2*	*10.0*	*268*	*290*	*16*	*53*
74	8	249	8.1	13.2	3.8	254	335	21	37
		265	*9.7*	*13.8*	*4.9*	*281*	*299*	*17*	.....
77	13	259	7.3	12.4	6.5	259	337	19	31
		254	*8.8*	*12.9*	*6.0*	*265*	*319*	*19*	*30*
78	7	249	6.3	12.4	2.8	258	322	20	38
		278	*7.0*	*12.6*	*9.7*	.....	*271*	*16*	*15*
80	8	251	4.3	11.8	3.8	238	352	22	50
		265	*11.9*	*12.2*	*4.1*	*268*	*315*	.....	*34*
32	5	257	4.7	.....	2.0	213.5	357	24	80
		270	*6.6*	.....	*2.5*	*230.5*	*418*	*18.6*	*30*
65 (moribund)	7	254	.....	9.8	1.4	267	347	22	73
		282	*8.7*	*12.7*	.....	*296*	*265*	*6*	*30*
75 (moribund)	6	267	10.2	14.4	3.7	256	342	20	27
		306	*8.7*	*15.6*	*4.8*	.....	*239*	*18*	.....

* In this and subsequent tables (Tables 2 and 3), any pair of figures indicate the value of the blood sample drawn immediately prior to (roman-face type) and at some specified time (second column) after the operation (italic-face type). mEq/l = milliequivalent per liter. mM/l = millimols per liter.

ceptible increases. There was a rise in potassium in all but one (No. 75) of those in which the determination was made. Calcium usually showed little change. One increase (No. 65) of 29 per cent is responsible for the average rise. Magnesium rose in all but one (No. 77) of the seven skates in which it was determined. However, the rise was marked in only three animals. Chlorides rose in all but one (No. 63) of the seven skates in which it was determined. Blood urea fell

TABLE 2

BLOOD CHANGES IN INTERRENALECTOMIZED SKATES: "ACTIVE"

No.	Days after Operation	Sodium (mEq/l)	Potassium (mEq/l)	Calcium (mEq/l)	Magnesium (mEq/l)	Chloride (mEq/l)	Urea (mM/l)	Erythrocytes (Volume per Cent)	Glucose (Milligrams per Cent)
28	5	244	8.7	.....	.....	246	.....	15	22
		261	*9.3*	*7.7*	*3.3*	*260*	*334*	*14*	.....
29	9	236	8.7	7.3	.....	249	334	17	58
		246	*11.0*	*10.7*	*4.2*	*279*	*299*	*17*	*8*
30	9	250	6.6	9.5	.....	248	315	14	38
		260	*11.8*	*10.4*	*9.3*	*288*	*307*	*14*	*38*
34	15	241	10.4	.....	.....	258	345	18.5	32
		249	*7.0*	*12.0*	.....	*271*	*314*	*14*	*15*
39	6	248	5.2	11.5	.....	250	357	17	24
		289	*10.3*	*10.6*	*5.5*	*265*	*319*	*15*	*7*
40	4	241	9.3	10.6	3.9	255	340	20	26
		250	*12.2*	*13.0*	*4.3*	*250*	*342*	.....	*13*
41	8	242	6.2	.....	.....	248	360	21	21
		256	*9.1*	*10.6*	*4.4*	*248*	*342*	*22*	*8*
47	9	243	7.3	10.4	5.0	243	.....	18	43
		274	*10.8*	*12.8*	*4.7*	*274*	*317*	*13*	.....
67	5	230	8.2	12.0	2.7	245	349	18	65
		237	*12.8*	*12.0*	*3.0*	*276*	*302*	*12*	*60*
69	5	246	7.3	12.0	.....	279	296	15	38
		255	*12.6*	*13.1*	.....	*284*	*297*	*15*	*47*
70	7	287	7.8	.....	.....	263	332	20	55
		271	*7.9*	*12.6*	*4.2*	*278*	*302*	*18*	*17*
25	7	242	7.5	5.6	6.6	244	342	16.5	46
		282	*13.8*	*8.6*	*8.8*	*288*	*302*	*14.5*	*8*
42	2	252	11.3	12.3	4.0	250	414	18.5	13
		270	*13.5*	*9.8*	*3.1*	*260*	*295*	*13*	*7*
7	7	234	3.6	.....	2.0	205	381	21.6	45
		240	*3.6*	.....	*2.9*	*208*	*371*	*20.5*	*42*
86	6	263	8.9	13.8	5.9	258	309	20	63
		266	*12.2*	.....	.....	*281*	*278*	.....	*58*
2*	14	.....	.....	.....	.....	.....	384	.....	.....
		.....	.....	.....	.....	.....	*380*	.....	.....

* *R. diaphanes.*

in all but one animal (No. 72). Blood sugar fell in all animals. In two the change was insignificant. The volume per cent of erythrocytes likewise fell in all animals. In one the fall was great, because of hemorrhage, as shown by blood in the perivisceral fluid.

so we failed to obtain second blood samples from them. However, we succeeded in obtaining a second sample in twenty-seven interrenalectomized skates. With progression of interrenal insufficiency the fish became less active, not only remaining in one spot most of the time but be-

TABLE 3

BLOOD CHANGES IN INTERRENALECTOMIZED SKATES: "MARKEDLY INSUFFICIENT"

No.	Days after Operation	Sodium (mEq/l)	Potassium (mEq/l)	Calcium (mEq/l)	Magnesium (mEq/l)	Chloride (mEq/l)	Urea (mM/l)	Erythrocytes (Volume per Cent)	Glucose (Milligrams per Cent)
22	5	242	5.9	6.0	5.9	253	.....	16	.....
		272	*7.6*	.....	.....	*286*	.....	*14*	.....
24	2	266	7.4	.....	.....	240	280	20	55
		246	*8.6*	.....	.....	*246*	*135*	*14.5*	*7*
31	3	232	7.0	.....	.....	256	277	17	88
		304	*14.0*	*6.8*	*1.0*	*300*	*220*	*10*	*43*
33	10	242	6.9	.....	.....	253	324	20	25
		248	*10.1*	*10.0*	*3.4*	*262*	*280*	*16*	*13*
66	5	260	10.0	11.2	2.8	265	322	23	61
		264	*12.4*	*12.8*	.....	*280*	*277*	*12.5*	*53*
68	8	245	6.0	12.4	.....	248	334	20	52
		284	*7.7*	*14.1*	*11.7*	*292*	*262*	*10*	*15*
139	5	266	7.2	.....	.....	251	328	26	30
		272	*7.2*	.....	.....	*279*	*283*	*19*	*17*
138	6	260	5.3	.....	.....	259	347	19	64
		288	*10.3*	.....	.....	*268*	*310*	*13*	*18*
140	3	270	5.1	.....	.....	257	334	18	76
		291	*9.6*	.....	.....	*276*	*255*	*14*	*83*
142*	4	279	8.1	.....	.....	264	333	22	98
		287	*7.2*	.....	.....	*309*	*248*	*19*	*54*
143*	2	273	5.2	.....	.....	266	308	18	106
		334	*8.6*	.....	.....	*338*	*202*	*8*	.....

* *R. eglanteria.*

The outstanding changes in the controls were decreases in urea, sugar, and erythrocytes of the blood and increases in the sodium and potassium of the plasma.

Interrenalectomized skates (*Tables 2 and 3*).—Many of our interrenalectomized animals died during the night, and

ing disinclined to move when stimulated. The change in spiracular movement was characteristic. It gradually decreased in rate and amplitude until it stopped in the completely relaxed position. Just before death the pectoral fins began to curl, and opisthotonous developed. The skin became dull in color and appeared poorly

nourished. Aside from the spiracular movement, there was nothing very distinctive in these changes. There was just a general sluggishness in all activities. At autopsy, if the insufficiency had become marked, the spiral valve might be congested or hemorrhagic.

Blood taken from interrenalectomized skates while they were quite active would be free from the changes that occur just preceding death, so that it is more likely from sham-operated animals except in the greater fall in volume of packed erythrocytes.

The data treating of the changes in certain blood constituents of sham-operated skates, and "active" and "markedly insufficient" interrenalectomized skates are summarized in Table 4. The data represent the percentage of changes occurring in the respective blood constituents as a result of the operative procedure indi-

TABLE 4

SUMMARY OF CHANGES SHOWN IN SHAM-OPERATED AND INTERRENALECTOMIZED SKATES
(Values in Per Cent)

Blood Constituents	(Sham-operated)			Interrenalectomized					
	1. Controls			2. "Active"			3. "Markedly Insufficient" (Inactive)		
	No. of Fish	True Mean*	S.D.†	No. of Fish	True Mean*	S.D.†	No. of Fish	True Mean*	S.D.†
Na	10	+ 4.1± 2.8	9.0	15	+ 5.7± 1.5	5.8	11	+ 9.3± 3.3	10.8
K	7	+41.3±23.7	62.6	15	+39.0± 0.9	36.3	11	+43.9±11.4	38.0
Ca	9	+ 5.2± 3.3	9.8	9	+15.2± 8.1	24.2	....	.....	.....
Mg	8	+79.0±34.5	96.8	6	+12.0±10.2	24.6	....	.....	.....
Cl	8	+ 6.5± 1.9	5.3	15	+ 7.1± 1.6	6.1	11	+11.8± 2.3	7.6
Urea	10	−10.2± 3.9	12.6	13	− 8.6± 2.1	7.7	10	−21.7± 2.9	9.3
Blood sugar	8	−40.2± 9.4	28.1	12	−43.2± 3.6	35.2	9	−46.8±10.0	29.9
Red cells	9	−20.7± 7.3	20.9	13	−12.0± 3.7	12.8	11	−31.5± 4.4	14.7

*True mean = arithmetic mean ± probable error of that mean.

†S.D. = standard deviation of mean.

to reflect the direct effect of the interrenalectomy. The results from the analyses in this group are shown in Table 2. The changes were similar to those found in sham-operated animals except that there was a greater increase in calcium and a smaller increase in magnesium in those animals in which the determination was made.

Blood taken from interrenalectomized skates in a late stage of insufficiency should show changes if they occur. The third group (Table 3) is composed of animals in this stage. Blood from these animals differed little from that taken

cated. The statistical characteristics of the data presented are the true mean (arithmetic mean ± probable error) and the standard deviation of the mean for each of the three groups of experimental animals. A statistical analysis of the summarized data is presented in Table 5. Here the differences between "active" interrenalectomized and sham-operated (control) skates (first column), as well as between "markedly insufficient" interrenalectomized and control skates (second column), are compared. For any set of data to be statistically significant their ratio (column headed "Diff./P.E.

Diff.") must be equal to, or greater than, 3. A perusal of these ratios indicates that only the difference between the values of plasma magnesium in "active" interrenalectomized and control skates is significant. However, in the light of the relatively few (six) fish employed, this apparently significant change in plasma magnesium must be treated with reservation.

The change in liver glycogen is apparently significant. There was a fall in interrenalectomized animals, as compared with the normal or sham-operated animals. The mean values were: interrenalectomized, 594 mg. per cent; normal, 1,674 mg. per cent; and sham-operated, 4,542 mg. per cent. The very high glycogen values shown by the sham-operated group are perhaps worthy of

TABLE 5

STATISTICAL ANALYSIS OF DIFFERENCES BETWEEN "ACTIVE" AND "MARKEDLY INSUFFICIENT" INTERRENALECTOMIZED AND SHAM-OPERATED (CONTROL) SKATES

Blood Constituents	"Active" Interrenalectomized—Controls		"Markedly Insufficient" Interrenalectomized—Controls	
	Difference*	Diff./P.E. Diff.†	Difference*	Diff./P.E. Diff.†
Na	1.6± 3.2	0.5	5.2± 4.3	1.2
K	2.3±23.8	0.1	2.6±26.3	0.1
Ca	10.8± 8.6	1.3	.....	.....
Mg	67.0±14.9	4.5	.....	.....
Cl	0.6± 2.6	0.3	5.3± 3.0	1.8
Urea	1.2± 4.4	0.3	11.5± 4.9	2.3
Blood sugar	3.0±10.1	0.3	6.6±13.7	0.5
Red cells	8.7± 8.2	1.1	10.8± 8.5	1.3

* Difference = difference between the designated arithmetic mean and its probable error.

† Diff./P.E. diff. = The ratio of the difference between two means divided by the probable error of that difference. When the ratio is three times or over, the data is considered to be statistically significant.

TABLE 6

LIVER GLYCOGEN IN SKATES

Normal		Sham-operated		Interrenalectomized	
Skate	Liver Glycogen (Milligram per Cent)	Skate	Liver Glycogen (Milligram per Cent)	Skate	Liver Glycogen (Milligram per Cent)
36	112	78	272	67	8
48	284	72	4,000	24	33
35	642	63	4,771	138	104
27	849	65	5,610	25	107
59	850	73	8,059	33	490
29	1,043			30	683
64	1,297			86	919
78*	2,467			40	1,030
63*	3,875			29	1,089
79*	5,322			39	1,475
Mean	1,674±508	Mean	4,542±1,274	Mean	594±166
S.D.	1,610	S.D.	2,850	S.D.	524

* Biopsy.

note. The mean value here is approximately three times that of the normal animals. On two of the sham-operated animals (Nos. 63 and 78) biopsies were performed, and these values are included in the normal group. It will be seen that in one case (No. 63) the liver glycogen actually increased, though, as stated under "Methods," these animals were fasting. In the other (No. 73), it fell. This will be commented upon further in the discussion.

In one interrenalectomized skate, the effect of injected glucose on blood sugar and on glycogen storage was determined. This animal was observed for 14 days after interrenalectomy and was very vigorous at the end of that time. Although it was trapped 3 days before the operation and had no food thereafter, it lost no weight. The normal blood urea was 384 mM/*l*. One week after the removal of the interrenals it had fallen to 362 mM/l, but 2 weeks later it had returned to approximately the normal level. At this time its normal blood sugar was determined, and it was given 1.5 cc. of 10 per cent glucose per kilogram (600 mg.) intravenously. Two smaller animals were run as controls, being given the same amount of glucose per kilogram of body weight. Blood-sugar determinations were made at intervals, as shown in Figure 1. It will be noted that one of the controls had an abnormally high initial blood sugar. Aside from this, the curve in the animal was normal. The curve for the interrenalectomized skate is normal except for a greater percentage rise over the initial value during the first 5 minutes. Thus the experimental animal showed a 640 per cent rise during this time, while control No. 15 showed a 142 per cent rise and control No. 1 a 240 per cent rise.

After determining blood sugar at intervals over a 15-hour period the animals were sacrificed and the livers analyzed for glycogen. The liver of the interrenalectomized skate (No. 2, weight 3,900 gm.) contained only 111 mg. of glycogen (110 mg. per cent), while that of control animal No. 1 (weight 1,100 gm.) contained 360 mg. (720 mg. per cent) and that of control No. 15 (weight 800 gm.) 732 mg. (2,928 mg. per cent).

DISCUSSION

Biedl (1913) interrenalectomized thirty-two members of the family Rajidae. He states that the first symptoms appeared at about the seventh or eighth day. Fourteen to 18 days after operation they became so weak that they could scarcely be induced to swim. At the latest, they died 3 weeks after operation. He fails to state the average survival time of the animals in this group. He says that, apart from violent hemorrhage and destruction of the renal tissue, the severing of the abdominal sutures and consequent prolapse of the viscera was the most frequent cause of death. One must assume that he may have lost a number of animals in this way. Kisch's (1928) animals, *Torpedo ocellata* and *T. marmorata*, survived 8–20 days after interrenalectomy. He presents data on only six animals. Dittus (1937), working on

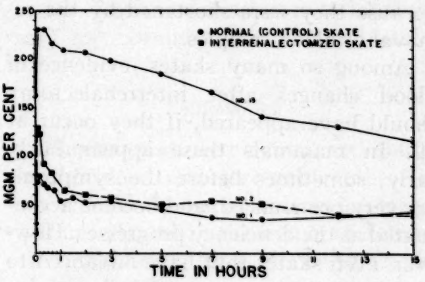

FIG. 1.—Sugar-tolerance curves following the intravenous injection of 1.5 cc. per kilo of 10 per cent glucose solution.

these species, obtained shorter survival, viz., 2–7 days.

We chose a species in which hemorrhage and renal injury could be avoided for the most part. However, we did find difficulty in preventing a pulling-out of the sutures. This was anticipated by taking the second sample of blood when the first sign of slipping of the sutures appeared. In this way, samples were obtained before sea water had entered the body cavity, as indicated by the low magnesium in the plasma.

Our survival times were not comparable to those reported by Biedl and Kisch because they were shortened by the removal of blood samples.

Among so many skates, evidence of blood changes after interrenalectomy should have appeared, if they occur at all. In mammals these appear fairly early, sometimes before the symptoms are very pronounced, and become accentuated as the deficiency progresses. However, even skates that have advanced to the state of almost complete inactivity fail to show characteristic modifications. The operation itself, without the interrenalectomy, can account for the observed changes. Indeed, the fall in blood urea and rise in plasma sodium can be accounted for by the inanition (Hartman *et al.*, 1941).

It has been pointed out (Hartman *et al.*, 1941) that some normal skates survive a relatively short time under even the best laboratory conditions. Occasionally they may survive many weeks. Undoubtedly, interrenalectomy shortens their lives. The temperature of the water is important. While we were able to keep the temperature low by refrigeration, our normal animals lived longer than they did when refrigeration was discontinued.

If the interrenal plays any role in the electrolyte metabolism of the skate, there is no evidence to support it in our observations. Likewise the blood-sugar changes indicate no interrenal responsibility. However, this would not test the relation to gluconeogenesis, because sham-operated animals also have a low blood sugar.

Our liver glycogen determinations suggest that the interrenal is a factor in gluconeogenesis in the skate. It will be noted that the three highest liver glycogens among the normal group were obtained from biopsies. In these cases, as stated under "Methods," only a small portion of one lobe was analyzed. Gregg (1933) has shown that liver glycogen varies considerably from lobe to lobe. The greatest variation found was ± 25.2 per cent. Even if the three biopsy determinations in our normal series are decreased by 25 per cent, the corrected average (1,524 mg. per cent) is still 157 per cent greater than the average of the interrenalectomized animals. This factor would have no influence on the sham-operated group, since all of these were determinations on whole livers.

The extremely high values found in the sham-operated animals were, at first, surprising. However, it is quite possible that the stress of the operation was a sufficient stimulus to the interrenals to produce overactivity of the secretory cells. This has been shown to occur in rats subjected to the stress of low barometric pressure (Evans, 1935).

Our results on sugar curves show that glucose injected into the interrenalectomized animal disappeared from the blood, as in normal animals, but, at the time of sampling the liver (15 hours after injection), was not stored in the liver, as in the controls. It may have been (1) excreted by the kidney, (2) stored in the muscle, or (3) oxidized by the tissues. We are unaware of any experimental work which would support the first supposition, namely, that the kidney of the ad-

renalectomized animal has a lower threshold for sugar than the normal kidney. The second possibility is likewise unsupported by experimental evidence. In the adrenalectomized dog, even when muscle glycogen was lowered by electrical stimulation, restoration from injected glucose was very slow, and never as complete as in normal animals (Dambrosia, Leloir, and Vovelli, 1933). It seems inconceivable, then, that the skate with a low liver glycogen should dispose of injected glucose by synthesis of glycogen in the muscle. Therefore, only the third supposition remains, i.e., that glucose is more rapidly oxidized by the tissues of interrenalectomized animals than by those of normal animals. For this, there is supporting evidence from mammalian experiments. Thus, Evans (1941) observed more rapid disappearance of intravenously injected glucose in adrenalectomized rats with ligated kidneys than in normals. Analyses of liver and muscle failed to reveal it. He therefore concluded that it had been oxidized. Unfortunately, facilities were not available for the analysis of muscle in this study, nor was it practicable to tie off the kidney. However, one of our controls (No. 15) is rather good evidence against the theory that sufficient sugar was being excreted to prevent its storage as glycogen in the liver. This animal (Fig. 1) had a very high initial blood sugar. The highest point on this control curve was more than twice that of the maximum for the interrenalectomized animal, and the curve remained considerably higher throughout; yet the control animal was able to store the largest amount of liver glycogen of the three. On the other hand, it might be argued that the blood-sugar curve of the experimental animal was not high enough to permit storage, had not control No. 1, with an even lower glucose tolerance curve, stored more than three times as much glycogen in its liver. The increased oxidation theory seems the most tenable. However, the only positive proof would be tissue metabolism studies with glucose added to the medium.

We must conclude from these results that the elasmobranch interrenal does not influence electrolyte metabolism. Thus, it does not produce the sodium factor. However, it appears to be necessary for normal carbohydrate metabolism.

SUMMARY

Plasma electrolytes, urea, and glucose in the blood and glycogen in the liver were determined in normal, in interrenalectomized, and in sham-operated skates (*R. erinacea*). After interrenalectomy, plasma sodium, potassium, magnesium, and chlorides rose, while urea, glucose, and erythrocytes of the blood decreased. Statistical analysis of the summarized data indicated no significant difference between the interrenalectomized and the sham-operated animals. This held true for animals in the last stage of insufficiency, as well as for interrenalectomized animals still active. There was no evidence that the interrenal plays any role in electrolyte metabolism. Although the blood-sugar changes indicated no interrenal responsibility, the fall in liver glycogen showed that the interrenal is necessary for normal carbohydrate metabolism.

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ERRATUM

USE OF PARA-AMINODIMETHYLANILINE: A CORRECTION

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AN ERROR concerning approximate concentrations of p-aminodimethylaniline used in the indophenol reaction in studies on oxidation-reduction pattern in living organisms requires correction. This error has appeared in four papers already published.[1] The reagent was obtained from the Eastman Kodak Company in small vials labeled 10 gm. or 25 gm., usually in liquid, sometimes in solid, form. In addition to the name of the chemical, the label carries the statement: "This product contains 5 per cent or more of an aromatic amine." Since the aniline is an aromatic amine, this statement was interpreted by the writer as meaning that the product as sold contained approximately 5 per cent of aniline. In the four papers cited, experimental concentrations of aniline were calculated on the basis of that interpretation. With continued use of aniline it was found that different samples varied widely in value for the indophenol reaction and that all gradually deteriorated more or less rapidly with the frequent opening of the vials to obtain a drop. With given concentrations of the altered aniline and α-naphthol the solution was more irritating, more toxic, the reaction differential became less distinct, there was more differential decoloration, and the animals died and disintegrated much sooner than with the same volume concentrations of the best liquid samples of aniline. In general, the solid product gave results much like those observed with the altered liquid.

[1] C. M. Child, Formation and reduction of indophenol blue in development of an echinoderm, Proc. Natl. Acad. Sci., 27:523, 1941; Indophenol reaction and reduction in living ovaries of *Drosophila hydei*, Physiol. Zoöl., 16:141, 1943; Gradient pattern in developing gill, leg, and balancer of *Triturus*, *ibid.*, p. 287, 1943; Oxidation-reduction pattern in development of a teleost. *ibid.*, p. 297, 1943.

Inquiries made of the Eastman Company concerning the aniline reagent and the difficulties encountered in its use brought the information that the statement on the label as to 5 per cent or more content of an aromatic amine means nothing as regards actual concentration but is merely required by law for all chemicals or solutions containing any amount of an aromatic amine from 5 per cent on. Moreover, the p-aminodimethylaniline prepared by the Eastman Company is not in a solvent, though it may be liquid, and "is certainly more than 5 per cent pure." Moreover, according to the information given me, the melting-point is not far from room temperature and the solid form is likely to be more nearly pure than the liquid. I am indebted to Mr. C. R. Lee, of the Chemical Sales Division of the Eastman Kodak Company, for his kindness in replying to my questions.

Since the aniline oxidizes rather readily and apparently is otherwise somewhat unstable, the actual concentrations of the effective reagent in the solutions used for experiment are not known with certainty. If the most satisfactory liquid samples obtained are 100 per cent pure, the experimental concentrations of the aniline reagent are approximately twenty times those given in the papers cited. Probably they are often considerably less than that. Concentrations of α-naphthol are approximately correct as given.

The error and uncertainty as regards actual experimental concentrations of aniline do not alter the significance of the oxidation-reduction gradient patterns demonstrated by the indophenol reaction. It has been noted repeatedly that the patterns are not essentially altered except in time of appearance, with great differences in concentration of one or both reagents, until concentrations are extremely low.

NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

VITAMINS AND HORMONES, VOLUME I,[1] EDITED BY ROBERT S. HARRIS AND KENNETH V. THIMANN

Reviewed by C. DONNELL TURNER
Northwestern University

THE studies upon metabolism and chemical co-ordination have been complicated far beyond the anticipation of the original investigators by the discovery of numerous transitional and unpredicted types of chemical reactions occurring in protoplasm. With the advancement of knowledge, the line of demarcation between vitamins, hormones, enzymes, etc., has become faint indeed. The title of this book suggests that there is some degree of relationship between vitamins and hormones. Among both classes of materials there is so much physiological and chemical variation that neither can be defined with accuracy. The vitamins usually are considered to act after the manner of catalysts, and some of them have been shown to constitute indispensable components of the enzyme systems which are concerned in the processes of cellular oxidations and reductions. In general, it appears that vitamins make possible many of the cellular changes which are included in the term "metabolism" and that the hormones function in adjusting and integrating these complicated metabolic reactions within the various tissues of the organism. The exact mechanism whereby hormones exert their effects remains obscure.

It is the purpose of this publication to collate and digest the enormous literature on vitamins and hormones which has accumulated during recent years. As pointed out by E. V. McCollum in the Foreword,

> We must increasingly depend upon our colleagues, who maintain mastery of specialized experimentation, to appraise for us the numerous contributions which they alone can interpret, sifting error from truth and assembling scattered data to make a connected account which places a body of related facts in proper perspective.

Since this is intended primarily as a reference book, all the chapters are thoroughly documented, and very extensive subject and author indexes are included at the end of the volume. Though most of the material is an analysis of published observations, many hitherto unpublished data are included, and, fortunately, various viewpoints in the interpretations of them are presented. The reviewer is not able to detect any central theme running through the volume, but this is hardly to be expected, since it is actually an encyclopedic account of special research fields prepared by different investigators. The editors anticipate the annual publication of additional volumes and express the hope that the series will serve to chronicle progress in these two fields and indicate the directions in which additional re-

[1] New York: Academic Press, Inc., 1943. Pp. xviii+452+illus. $6.50.

searches would be most fruitful and useful.

In Volume I there are eight chapters dealing with dietary factors and two with hormonal compounds. The chemistry and nutritional significance of choline are discussed by C. H. Best and C. C. Lucas. Their treatise includes the chemical properties, synthesis, assay, intermediary metabolism, and physiological importance of this compound. It is concluded that there is no proof that the pancreas secretes a second internal secretion, termed "lipocaic" by Dragstedt and his collaborators, but that there is proof that this tissue does contain an unidentified lipotropic agent in addition to choline. The contribution by George R. Minot and Maurice B. Strauss, entitled "Physiology of Antipernicious Anemia Material," is an excellent summary of our knowledge of this principle.

Five chapters deal rather definitely with the physiological significance of vitamins. H. H. Mitchell summarizes the chemical and functional relationships existing between vitamins and amino acids, their derivatives and aggregates in the animal organism. That vitamin-protein combinations facilitate many protoplasmic transformations is convincingly indicated by the elucidation of the role of flavoproteins in biological oxidations, the role of the carotenoid proteins in photoreception, and the role of ascorbic acid in the formation of collagen. The chapter by George Wald, entitled "The Photoreceptor Function of the Carotenoids and Vitamins A," will be of special interest to theoretical biologists. After presenting and evaluating available evidence, the author concludes that the photoreceptor systems of both plants and animals are linked in a common dependence upon carotenoids. These pigments are considered in relation to their vitamins A, and especial attention is devoted to the functional evolution of these compounds in the animal kingdom. The results obtained in the attempt to assay the vitamin content of different plant and animal tissues are presented by Roger J. Williams in a chapter entitled "The Significance of the Vitamin Content of Tissues." Our knowledge of growth factors in Protozoa is ably discussed by Richard P. Hall. It is pointed out that some of the Protozoa are capable of thriving in media free from growth factors, whereas others can be grown only in media containing these agents. It is not known whether or not any protozoan is capable of synthesizing vitamins. Research in this field has been confined to only a few laboratories, and the sparsity of conclusive data is often traceable to the utilization of inadequate technical procedures. The chapter by Norman Jolliffe and Rita M. Most is an appraisal of nutritional status in the human subject and should be of particular interest to those in the field of clinical medicine. The importance of recognizing preclinical states of malnutrition in individuals and population groups is emphasized.

The contribution by John R. Loofbourow describes and evaluates the physical methods which may be employed in the identification and assay of vitamins and hormones.

The last two chapters of this volume are devoted to hormones. Gregory Pincus and William H. Pearlman discuss the intermediate metabolism of the sex hormones. The steroid hormones of the adrenal cortex are treated by T. Reichstein and C. W. Shoppee. This chapter is an excellent review of the chemical features of these compounds.

In this volume a vast amount of current information has been assembled and

made accessible to professional readers. Though a high degree of unity cannot be expected in a composite volume of this type, the reviewer feels that it might have been improved by a more logical grouping of the chapters.

PHYSIOLOGICAL REGULATIONS[1] BY EDWARD F. ADOLPH

Reviewed by RUDOLF T. KEMPTON
Vassar College

THIS book is not a review but a well-reasoned, quantitative analysis of processes of regulation. Much of the information on which it is based appears here for the first time and is added to a reinterpretation of much of the older literature and to earlier work by Adolph and his co-workers. Adolph clarifies the broad field of regulation and correlates a large number of isolated studies by considering regulations quantitatively, by quantitative comparisons between species, and by using equilibration diagrams to represent dual processes in a given species.

The central theme of the book is the regulation of water content; but, in addition, the author considers other types of regulation, such as that of heat, heart rate, blood pressure, blood flow, and regeneration. The book considers regulatory mechanisms of representatives of the animal kingdom extending from the Protozoa to man. With the mechanics of this regulation subject to enormous complexities of variation, the author finds, nevertheless, that there are fundamental resemblances common to all types of regulation and to all kinds of animal organisms.

It is the variation rather than the constancy of living material which has interested the author. Instead of accepting the dictum that the *milieu intérieur* of Claude Bernard is remarkably constant, the author has been interested in the deviations from that average state, the amount of disturbance which can be tolerated, the kinetics of recovery from both positive and negative loads, the variety of mechanisms and routes involved in this recovery, and the correlation between the phenomena related to one type of regulation and those changes found in another type of regulation. He pictures the living organism as containing a large number of regulatory mechanisms, each for a specific property of the animal, with all the mechanisms being mutually compatible.

In general, Adolph finds that the variability of any component is limited in extent and that the greater the deviation from the norm, the greater the velocity of recovery. This applies to all the mechanisms studied. Any animal in a state of water balance has its intake and output in equilibrium. Significantly, in most cases this occurs at a low point in the velocity of possible intake and output. It is this fact which explains the ability of many animals to recover rapidly from heavy positive or negative loads.

The rate of recovery from loads for any component is highly variable from animal to animal and from one component to another in the same animal. The velocity quotients of these recoveries may differ by factors as great as 10^5. Perhaps it is this enormous variation which has tended for so long to conceal the fundamental similarities of the processes. But, whatever the rate, "animals preserve their constitutions like

[1] Lancaster, Pa.: Jacques Cattell Press, 1943. Pp. xvi+502+186 figs.+46 tables. $7.50.

themselves, within the limits of variation that characterize the normal, either by preventing disturbances from occurring, or by compensating for each actual and incipient departure from normal."

Whether or not one agrees with every interpretation, whether or not the test of time supports all the generalizations, the book appears to be a valuable contribution to biological literature. As a result of its quantification and clarification of the field, its demonstration of gaps in existing data, its extensive Bibliography, and its suggested lines of future study, the book should be very stimulating to future investigation.

BIOLOGICAL SYMPOSIA, VOLUME X: *FRONTIERS IN CYTOCHEMISTRY*[1] EDITED BY NORMAND L. HOERR

Reviewed by ERIC G. BALL
Harvard University

THIS volume dealing with the physical and chemical organization of the cytoplasm is certainly well worthy of the title *Frontiers in Cytochemistry*. There is much that is new and stimulating between its covers. It consists of fourteen articles written by different investigators who are among the poineers in their fields. The central theme running through the volume stems from Dr. R. R. Bensley, to whom the book is dedicated. This is the application to the cell of the principle of separating separable things first, before proceeding to the analysis of the cell's many components.

Dr. Bensley says: The alternative method of mixing all the constituents of the cell as thoroughly as possible and then obtaining from the mixture substances in pure form by skillful chemical procedures has yielded brilliant results in the field of hormones, enzymes, nucleic acids, etc., but has left us in ignorance of the protein structural substrate of the cell and of the distribution and spatial relationships of the active substances.

Here in this volume one may learn the techniques that enable one to separate nuclei, mitochondria, glycogen, or submicroscopic particulates from the cell. Articles by A. Lazarow, A. Claude, N. L. Hoerr, and R. R. Bensley not only describe these techniques but also record some of the observations made upon the composition of these separable components of the cell. The separated mitochondria, for example, are yellow in color and possess a variety of enzymatic activities. The separated submicroscopic particulates are cherry-red in color, and their lipide content is nearly 50 per cent of their dry weight. Both fractions show marked respiratory activity. Some of the oxidative enzymes concerned in this respiratory activity have been studied by A. Lazarow and E. S. G. Barron; and the chapter by Barron on "The Application of Biological Oxidation-Reduction Reaction Systems to the Study of Cellular Respiration" serves to show the reader the great possibilities that still exist for further research along these lines. Mirsky and Pollister contribute a chapter on the fibrous nucleoproteins of chromatin. Their evidence indicates that chromatin is largely, if not entirely, a complex of a highly polymerized desoxyribose nucleic acid with a basic protein of either the protamine or the histone type. The chromosomes may, therefore,

[1] Lancaster, Pa.: Jacques Cattell Press, 1943. Pp. vii+334. $3.50.

be 100 per cent nucleoprotein. Mirsky and Pollister ask: "Can we find in nucleohistone the chemical basis of heredity?"

The inorganic composition of protoplasm is dealt with in three articles. The results of the application of purely chemical methods to the analysis of tissues are described in a chapter by O. H. Lowry entitled "Electrolytes in the Cytoplasm." Aided by the so-called deductive histochemical method, these results indicate the striking difference in inorganic composition of the intracellular and extracellular phase of tissues. They also indicate the marked variation in the electrolyte pattern of the intracellular phase of various tissues. However, in spite of the excellent evidence which indicates that potassium is the predominant cation of the intracellular phase, it is interesting to note that the separable components of the cell mentioned above are purified during their separation by washing in 0.9 per cent sodium chloride! Certainly, it would seem that, if the unchanged enzymatic activities of these intracellular components are to be measured, they should be afforded at all times during their isolation an ionic environment more closely resembling that found within the cell. This aspect of the subject is well presented in the chapter by Robert Chambers, entitled, "Electrolytic Solutions Compatible with the Maintenance of Protoplasmic Structures." The antagonistic action of salts in the maintenance of the cell membranes furnishes particularly striking evidence of the influence of electrolyte pattern on the integrity of cell structures. A balance of sodium versus calcium would appear to be essential for maintaining the extraneous coat of the cell, while a balance of sodium versus potassium is needed for the integrity of the plasma membrane. The interesting results obtained in the attempt to localize the various inorganic components of the cell are reported by G. H. Scott in a chapter entitled "Mineral Distribution in the Cytoplasm." Emission electron micrographs obtained by this author indicate that either calcium or magnesium or both (since the method does not distinguish between the two) are concentrated in the nucleus, the cell membrane, the anisotropic disks of striated muscle, the zymogen granules of the pancreas, and in certain definite locations in some epithelial cells. Little, if any, calcium plus magnesium is found in the areas between these cytoplasmic differentiations, though photographs of microincinerated tissue sections show that these areas do contain an abundance of mineral material. These findings furnish much food for thought concerning the relationship of ionic pattern to cell structure and activity.

Information on cellular components obtained by use of the ultracentrifuge, electron microscope, and by electrophoresis is presented in three chapters by H. W. Beams, F. O. Schmitt and his co-workers, and K. G. Stern, respectively. Space does not permit mention of the interesting findings obtained by these authors with these modern tools.

Two chapters are included which deal with the changes in cellular components that occur as the cell undergoes a pathological change. These are a chapter by E. V. Cowdry entitled "Experimental Epidermal Methylcholanthrene Carcinogenesis in Mice," and one by I. Gersh and D. Bodian on the "Histochemical Analysis of Changes in *Rhesus* Motoneurons after Root Sections."

After reading this book, the reviewer could not help wondering whether this approach to the study of the cell might not be the ideal method to employ in teaching both histology and biochemis-

try to students. Certainly, the present methods of teaching these subjects leave much to be desired as far as the integration of the knowledge that both sciences have to offer the student. At any rate, there is much in this book that should stimulate both the student and the teacher in either field.

VERTEBRATE PHOTORECEPTORS[1] BY SAMUEL R. DETWILER

Reviewed by L. B. AREY
Northwestern University Medical School

THIS moderately sized book is one in the series of "Experimental Biology Monographs." As the title suggests, the author has largely limited his presentation to the structure and activities of the rod, the cone, and the pigment cells of the retina. By so doing he does not invade the field covered by the intensive treatise of Polyak (*The Retina* [1941]); there is likewise only partial duplication of some of the topics more exhaustively treated by Walls (*The Vertebrate Eye* [1942]). Professor Detwiler, though presenting no small amount of detailed information, aims rather at the biologist who is not professionally concerned with the photoreceptive apparatus but who wishes to know its present status as an adaptive visual mechanism. To indicate the scope of the topics discussed, recourse may be made to an itemization of chapter contents.

1. *The vertebrate eye.*—An introduction in which the plan of the ocular bulb (mostly human) is set forth. An oddity is the devotion of a full page to the well-known Scottish tale of an illegitimate child who was born with the name of its presumptive father lettered in one iris and the birth date in the other. An "extraordinary case" indeed!

2. *The retina.*—Not described in the previous chapter as a constituent tunic of the bulb, it is treated here with proper emphasis on Polyak's analysis and synthesis of the conducting and integrating system of neurones.

3. *The structure of visual cells.*—Included is a summary of the difficulties encountered in assigning certain atypical visual cells to the rod or the cone category. Emphasis is placed on the criteria advanced by Kolmer through the use of differential staining.

4. *Differentiation of retinal elements.*—The development of the visual cells receives major attention. Arguments are marshaled against the claim that cones are specialized rods or that cones are rods arrested in development.

5. *Retinal structure and animal habits.*—A citation of the correlations existing between the kinds and ratios of visual cells found in any retina and the habits (diurnal, nocturnal, or both) of that animal. Walls's theory that cones have transmuted into rods in many nocturnal animals is presented, but the author still prefers to differentiate between rods and cones on a morphological basis.

6. *Photomechanical responses in the retina.*—A résumé of the capacities of rods, cones, and retinal pigment to undergo positional changes in light and darkness. Some other influences, such as temperature, are included as well.

7. *On the significance of photomechanical movements.*—A review of the possible usefulness of positional shifts in the visual receptors and retinal pigment in the furthering of efficient vision. No one theory is deemed sufficiently inclusive

[1] New York: Macmillan Co., 1943. Pp. x+184; 110 figs. $4.00.

to explain all the responses to light, temperature, etc., on an adaptive basis. The most conclusive evidence, showing how the several responses to bright and dim light in fishes and amphibians correlate with the duplicity theory, is, however, omitted.

8. *The relation of retinal structures to visual acuity.*—This chapter deals with the size and density of the visual elements in various animals, with the central area and different types of fovea developed in it, and with the neuronal connections within the retina that further the resolution of details.

9. *On the supposed evolutionary relationships and the functional significance of the fovea.*—The correlation between foveal development and the ability to perform extensive ocular movements is accepted; but conjugate movements, binocular vision, and partial decussation of the optic tracts are not necessarily implied. The author demurs against Elliott-Smith's view that the presence of a macula has led to marked evolutionary advances in the portions of the brain concerned with vision. Walls's recent interpretation of the fovea as a mechanism for enhancing the resolving power of the retina, not for mere optical thinness and homogeneity, is given sympathetic consideration.

10. *Retinal photopigments.*—An exposition of the properties of the rhodopsin and porphyrosin of the rod and of the iodopsin of the cone. The relations of these carotenoid derivatives to the vitamins A (as both precursor substances and decomposition products) are reviewed on the basis of Wald's important disclosures. The author identifies the Kolmer droplets, seen abundantly alongside dark-adapted rods, as retinene—the decomposition product intermediate between vitamin A and visual purple.

11. *Vitamin A deficiency and the retina.*—A timely digest of the dependence of vision on vitamin A, of the effect of its deficiency on physiological thresholds (night blindness), of the breakdown of rods following prolonged avitaminosis A, and of rod repair after return to an adequate diet.

This book carries one hundred and ten excellent illustrations, several extensive tables, a valuable Bibliography, and adequate author and subject indexes. It contains some original data, not published hitherto, of use as source material to the specialist. Professor Detwiler can be congratulated in having produced a readable, and at the same time authoritative, book in a field which owes much to his own investigative activities.